

Biochemical and Immunological Studies on β_2 -Microglobulin

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Abstract β_2-microglobulin (β_{2m}) was purified from the urine of rabbits with sodium chromate-induced proteinuria. The isolation schedule included ultrafiltration, gel chromatography, preparative electrophoresis, and ion-exchange chromatography. Some physicochemical properties of the protein were determined. The occurrence of β_{2m} in serum and in blood cells of various mammalian species was investigated. At the surface of rabbit and guinea pig lymphocytes β_{2m} was detected in association with class I transplantation antigens whereas guinea pig Ia antigens did not seem to have a β_{2m} subunit. Rat erythrocytes carry class I transplantation antigens and were also found to express β_{2m}. No β_{2m} was, however, detected on guinea pig, human, or rabbit erythrocytes. Since these cells are also devoid of transplantation antigens, the results indicate that β_{2m} and the heavy chain of these antigens are expressed together. Antibodies against β_{2m} were shown to be immunosuppressive by causing a moderate but significant prolongation of the survival of rat heart allografts. In immunodiffusion experiments antibodies to rabbit β_{2m} precipitated other mammalian β_{2m}'s and in cytotoxicity tests these antibodies lysed various mammalian and chicken lymphocytes. The results indicate that β_{2m} has been conserved during evolution. This was further emphasized utilising an assay based on the interaction between class I transplantation (AgB) antigens and human β_{2m}. Thus, fish sera were found to contain material capable of impeding this interaction. Aggregates of β_{2m} were bound to all group A and several group C and G streptococcal strains. The binding to group A strains seems to be mediated by M proteins; cell surface proteins of group A streptococci, which are important antiphagocytic and pathogenicity factors. The interaction between human β_{2m} and bacterial cell surface proteins could be of significance to the host-parasite relationship and might represent a reminiscence of primitive recognition mechanisms.		
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BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON β_2 - MICROGLOBULIN

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To

KRISTINA, HENG and ANNA

This thesis is based on the following papers which are referred to by their Roman numerals.

- I Lars Björck and Ingemar Berggård. Studies on β_2 -microglobulin in the rabbit. *Molecular Immunol.* (in press).
- II Lars Björck, Bo Åkerström, and Ingemar Berggård (1979). Occurrence of β_2 -microglobulin in mammalian lymphocytes and erythrocytes. *Eur. J. Immunol.* 9, 486-490.
- III Lars Björck, Ragnhild Cigén, Barbro Bergggård, Bengt Löw, and Ingemar Berggård (1977). Relationships between β_2 -microglobulin and alloantigens coded for by the major histocompatibility complexes of the rabbit and the guinea pig. *Scand. J. Immunol.* 6, 1063-1069.
- IV Lennart Lögdberg, Lars Björck, and Ragnhild Cigén (1980). Heterologous interaction between β_2 -microglobulin and AgB antigens. *Transplantation* 30, 233-235.
- V Lars Björck, Peter Konrad, Bo Husberg, and Ingemar Berggård (1977). Prolonged survival of heart allografts in rats treated with antisera to β_2 -microglobulin. *Transplantation* 23, 383-386.
- VI Göran Kronvall, Erling Myhre, Lars Björck, and Ingemar Berggård (1978). Binding of aggregated human β_2 -microglobulin to surface protein structure in group A, C, and G streptococci. *Infect. Immun.* 22, 136-142.
- VII Lars Björck, Stanisława Tylewska, Torkel Wadström, and Göran Kronvall. β_2 -microglobulin is bound to streptococcal M protein. *Scand. J. Immunol.* (in press).

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ABBREVIATIONS

β_2m	- β_2 -microglobulin
MHC	- Major histocompatibility complex
H-2	- Mouse MHC
HLA	- Human MHC
GPLA	- Guinea pig MHC
RLA	- Rabbit MHC
AgB	- Rat MHC
HLA-A, -B, and -C antigens, H-2K, H-2D, and H-2L antigens, GPLA-B and GPLA-S, RLA, and AgB antigens	- Classical transplantation (class I) antigens coded for by the correspon- ding HLA, H-2, GPLA, RLA and AgB loci.
I-region	- Immune response region within the MHC.
Ia antigens	- Immune response associated antigens coded for by loci within the I-region.
SDS - PAGE	- Polyacrylamide gel electrophoresis in sodium dodecyl sulphate
RIA	- Radioimmunoassay
MLR	- Mixed lymphocyte culture reaction

β_2 - MICROGLOBULIN: AN INTRODUCTION

Purification of β_2m

More than twenty years ago, Ingemar Berggård began to investigate macromolecules present in human urine. The work was originally focused on proteins, glycoproteins, and mucopolysaccharides in normal urine. This was the subject of Ingemar Berggård's thesis in 1961. Normal urine contains a large number of proteins (for further information and references see ref. 75). Some of these are derived from the urinary tract, but most originate from plasma. These latter proteins are filtrated through the glomerular membrane which discriminates sharply between plasma proteins of different molecular sizes. Accordingly, the glomerular fluid represents an ultrafiltrate of plasma and primarily contains small size proteins. Under normal conditions these proteins are almost completely reabsorbed in the proximal renal tubules. Patients with tubular disorders, however, excrete low molecular weight plasma proteins in large quantities. Since proteins belonging to this group are usually only trace components in normal plasma, urine from patients with tubular proteinuria is an excellent source for the isolation of plasma proteins of small size.

Ingemar Berggård published a preliminary report concerning a previously undescribed low molecular weight protein isolated from the urine of patients with tubular malfunction in 1965 (6). Three years later a detailed study was published on the purification and properties of this protein, which was called β_2 -microglobulin (β_2m) (7). The isolation procedure included ultrafiltration, zone electrophoresis, gel chromatography, and ion-exchange chromatography. This original procedure was later simp-

lified (5). Urine has consistently served as the best primary source for purification of large amounts of β_2m . The urine specimens were obtained from patients suffering from various tubular disorders. Apart from human β_2m , homologues of the protein have been isolated from several other species. In dog, rabbit, guinea pig and rat, urine from animals with experimentally induced proteinuria was used as starting material (8,17,71,112) whereas murine β_2m was isolated from sodium thiocyanate extracts of spleen and liver (83). Bovine β_2m was purified from milk (45), and a possible candidate for chicken β_2m was separated from serum (128).

Physicochemical properties of β_2m

Table 1 gives some general physicochemical properties of human β_2m . β_2m is a single polypeptide chain devoid of carbohydrate. It contains two half-cystinyl residues which are involved in an intrachain disulfide loop formed by 57 amino acid residues (20,38). This and other properties, such as sedimentation coefficients, Stokes' radii, molecular weights, and frictional ratios are shared between β_2m and immunoglobulin domains. Furthermore, investigations of the amino acid sequence of β_2m have revealed a significant homology with immunoglobulin molecules. Smithies and Poulik determined 44 of the first 46 residues of human β_2m (113). Computer comparison with the amino acid sequence of the heavy chain of immunoglobulin Eu (Ig G1) indicated a close relation between the two molecules. These studies were confirmed and extended by Peterson et al. (95) and Cunningham et al. (20). Analysis of the entire amino acid sequence of β_2m demonstrated sequence homology to the constant portion of immunoglobulin G light chain and to the three constant domains of the heavy chain. The homology with the C_H3 domain was found particularly outstanding (28 identical residues). The degree of homology was further emphasized by the fact that in 10 out of 11 positions where the four constant Eu domains all ha-

TABLE 1. Physicochemical properties of human β_2m ^a

Stokes' molecular radius, r_s	1,6 nm
Sedimentation coefficient, s_{20w}^0	1,6 S
Apparent diffusion coefficient, D_{20w}	133 $\mu m^2/s$
Frictional ratio, f/f_0	1,16
Molecular weight ^b	11,815
Free sulfhydryl groups	nil
Carbohydrate	nil
Absorption coefficient at 280 nm (pH 7,0)	$1,98 \times 10^4 M^{-1}cm^{-1}$

a Data taken from (7,51)

b Calculated from amino acid composition (7).

ve identical residues, these were also identical for β_2m . From these sequence data, it was suggested that β_2m represents a free immunoglobulin domain and that β_2m might have evolved from the same ancestral gene that gave rise to immunoglobulin light and heavy chains (95).

Clear-cut crystallographic data are still not available for β_2m . The multiple physicochemical properties shared between β_2m and immunoglobulin domains, however, make it reasonable to believe that the tertiary structure of these molecules will be found to be related. If true, the "basic immunoglobulin fold", which is an outstanding property of all the immunoglobulin domains, will characterize also the tertiary structure of β_2m . At a functional level, such a prediction is supported by reports sugges-

ting that β_2m can bind to lymphocyte Fc receptors (molecules with affinity for the C_{H3} domain of immunoglobulin G (129)) and, similar to the C_{H2} domain, interact with the first component of complement (93).

Despite the structural features shared by β_2m and immunoglobulins, antisera to immunoglobulins (G, A, M, D, E, kappa or lambda light chains, J chain, or secretory piece of immunoglobulin A) were initially not found to crossreact with β_2m and, conversely antisera to β_2m , did not react with immunoglobulins (7,113). Immunological cross-reactivity has later been described between β_2m and κ -light chains (42), and in a recent work by Vincent and Revillard (124), the authors used complement dependent lymphocytotoxicity and a sensitive antigen binding assay to detect cross-reactivity between β_2m and immunoglobulin G.

Synthesis and catabolism of β_2m

β_2m was originally found in urine and other biological fluids (7). In 1972 the protein was reported to be present at the cell surface of leucocytes (95), and the same year, β_2m was found to be produced by normal and phytohaemagglutinin stimulated lymphocytes (10). In long-term lymphoid tissue culture cell lines, β_2m and immunoglobulins were secreted independently, thereby indicating that the genes coding for the proteins are under separate regulatory mechanisms (49,86). Nilsson et al. (86,87) and Evrin and Nilsson (29) investigated a large number of cell lines of different histogenetic origin, and apart from two lines (the RPMI-8226 line derived from a patient with myeloma, and the Daudi line established from a Burkitts' lymphoma) all were found to secrete β_2m . The results suggested that all non-malignant, nucleated cells are capable of synthesising β_2m . Greatly increased serum levels of β_2m were reported by Bernier et al. (9) and Peterson et al. (94) in patients with renal failure and in bilateral-

ly nephrectomised patients. These observations indicate that $\beta_2\text{m}$ is catabolised mainly in the kidney.

$\beta_2\text{m}$; a protein of the cell membrane

The importance of plasma membranes in biological processes, such as, for instance, cell differentiation, cell-to-cell interaction, and transport of various nutrients, has acquired growing attention during the last decade. With this background the scientific interest in $\beta_2\text{m}$ was much increased when it was realised that the molecule was present at the cell surface. Today, $\beta_2\text{m}$ is perhaps the best characterised protein of the plasma membrane.

In comparing the amino acid sequence of immunoglobulin domains with the sequence of $\beta_2\text{m}$, Peterson et al. (95) found the most pronounced homology with the $\text{C}_{\text{H}}3$ domain, which was known to have affinity to cell surfaces. This information provided one of the incitements for these investigators to search for $\beta_2\text{m}$ at the cell surface. Employing radiolabelled antibodies against $\beta_2\text{m}$, the protein was, in fact, detected on the surface of leucocytes (95). Subsequent studies demonstrated that $\beta_2\text{m}$ was present on B and T lymphocytes, polymorphonuclear cells, platelets, and cells from a variety of tumours (28,30,100). The work by Nilsson et al. (87) on the synthesis of $\beta_2\text{m}$ also showed that the investigated cell lines of different histogenetic origin not only secrete $\beta_2\text{m}$ but also express the protein at their surface. Moreover, the presence of $\beta_2\text{m}$ has been reported on human spermatozoa (32) and using an indirect fluorescent method and tissue sections, Governa and Biguzzi (43) found strong fluorescence in lymph nodes, and palatine tonsils. Stomach, small intestine, and appendical tissue displayed positive reaction only in columnar epithelium, and endometrial tubular glands of the uterus also fluoresced. Negative results were obtai-

ned with kidney, urinary bladder, prostatic gland, liver, lung, thyroid, skeletal muscle, myocardium, ovary, and placenta. The authors stress, however, that the negative results do not necessarily exclude the presence of β_2m in these tissues, but rather indicate that cells derived from extra-embryonic mesenchyme are especially rich in β_2m . However, there are two normal human cell kinds that apparently lack β_2m , namely the mature erythrocyte and the cells of the trophoblastic layer of the placenta (28,31). Until proved otherwise, it seems plausible that all other normal cells both synthesize and carry β_2m at their surface.

In 1937, Gorer found that the immunological response in mice against transplanted tissue, leading to the rejection of the tissue, was controlled by a single chromosome (41); and in 1965, the corresponding observation was made in man (21,107). The chromosomal segment, the major histocompatibility complex (MHC), was called the H-2 region in mouse and the HLA complex in man. From a genetical and functional point of view, the MHC can be divided into regions. Thus, the I-region (HLA-D/DR in man) controls immune responsiveness against defined antigens (77), cell-to-cell interactions (for a review see ref. 53), and susceptibility to virus-induced tumours (69), and various other diseases (for a review see ref. 13). Other parts of the MHC harbour structural genes for complement factors (see ref. 62). The classical transplantation antigens (H-2K, H-2D, and H-2L in mouse and HLA-A, -B, and -C in man) finally are MHC gene products that serve as target molecules for cytotoxic T-lymphocytes (for reviews see refs 23,55).

Cell bound HLA antigens (for brevity HLA-A, -B, and -C antigens are referred to as HLA antigens in the following text) can be solubilized by limited proteolysis (108). In 1973, papain-solubilized HLA antigens were found

to be composed of two, non-covalently linked, polypeptide chains. One was carrying the serological activity, the other had no serologically detectable HLA antigenic determinants (18,80). The molecular weights of these polypeptides were estimated at about 30,000 and 10,000, respectively. Nakamuro et al. (81) found that the small HLA subunit showed great similarity to β_2m in amino acid composition, behaviour in gel filtration, and isoelectrofocusing experiments and on polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE). Ultimate proof that the two polypeptides are identical was presented by Tanigaki et al. (116). Using anti- β_2m antibodies, they demonstrated immunological identity between the molecules (116). Moreover, the amino acid sequence of the first 24 residues of the light HLA chain was found to be identical with the corresponding sequence of human β_2m . Independent of these investigations and using another experimental approach, Peterson et al. (96) also clearly demonstrated that the small HLA subunit is β_2m . HLA antigens were solubilized with papain and further purified by ion-exchange chromatography and gel chromatography. Fractions from the gel chromatography were analysed for β_2m by a solid phase radioimmunoassay (RIA). Two peaks with molecular weights of about 40-50,000 and 10,000 were detected. The first was found to contain all HLA antigenic activity, the second contained only β_2m . Purified HLA antigens were also labelled with ^{125}I and then subjected to SDS-PAGE. Three radioactive peaks were obtained. The fastest migrating zone belonged to β_2m , and the slowest had a molecular weight of 33,000. Anti-HLA antisera were used to precipitate the radiolabelled material. Two peaks (12,000 and 33,000) were obtained on SDS-PAGE. In reconstitution experiments with the two HLA chains, further evidence was given that the low molecular weight subunit of HLA antigens is β_2m . Such evidence was also published by Grey et al. (44).

At the cell surface, immunofluorescence experiments also showed a physical association between HLA antigens and β_2m (101). Such investigations also suggested that the number of β_2m molecules exceeds the number of HLA antigens, as β_2m only incompletely co-capped with HLA antigens (114). In mouse, β_2m was found in association not only with H-2K and H-2D antigens (82,104,110) but also with thymus leukemia (TL) and Qa antigens (78,91,115,126). TL antigens are expressed on thymus cells of some inbred mouse strains and on certain leukemia cell lines. The genes coding for these molecules are situated close to the H-2 complex. The Qa locus comprises a newly defined genetic region between H-2L and TLa, specifying lymphoid cell surface molecules (35). An important question raised by the association between β_2m and these various molecules coded for by genes within or close to the major histocompatibility complex was whether also the β_2m gene could be found in this area. The work of Goodfellow et al. (39) demonstrated that it could not. Whereas HLA is on chromosome 6 the β_2m gene was found on chromosome 15.

The isolation of tumour-specific antigens has been the subject of many investigations; perhaps the discovery that β_2m is a subunit of TL antigens raised expectations that additional tumour antigens were to be found in association with β_2m . Both oestrogen-induced and virus-transformed cells have, indeed, been reported to express tumour-specific antigens associated with the complex formed by β_2m and the heavy chain of the major histocompatibility antigens (16,59). Also other investigators studied putative tumour-specific antigens isolated from various tumours. Some have detected β_2m linked to these molecules, whereas others failed to find any such association (14,118).

In this survey of membrane proteins associated with β_2m , the histocompatibility Y-chromosome antigen (H-Y) should also be mentioned. This evolutionary highly conserved molecule was found to be responsible for the rejection of skin grafts, transplanted from male to female mice belonging to the same inbred mouse strain. The H-Y antigen is present on the surface of cells of the heterogametic sex, and also seems to be an inducer of this sex (for a H-Y review see ref. 127). In a work of Fellous et al. (33) H-Y antigen was reported to co-cap with β_2m but not with HLA antigens on human lymphoid cells. This observation perhaps indicates that β_2m and/or molecules associated with β_2m are involved in embryonal differentiation.

Some biological properties of β_2m

β_2m has been reported to bind complement. This binding is increased if aggregates of β_2m are used (93). Moreover β_2m promotes macrophage-rosetting of sheep blood cells (93). Human β_2m has cytophilic properties and is bound to rodent lymphocytes (1) and to human PHA treated lymphocytes (47). The heavy HLA chain has been claimed to lose most of its alloantigenicity when separated from β_2m , indicating that the HLA molecule is perhaps dependent on β_2m for its native configuration (67). The Daudi cell line does not produce β_2m but synthesizes HLA antigens (92). At the cell surface, however, there are no, or only few, HLA heavy chains (99). This probably means that β_2m is necessary for the cell surface expression of HLA antigens.

Antibodies to β_2m impede the response in mixed lymphocyte culture reactions (MLR) (3,70). Lindblom et al. (70) were able to block the MLR with Fab' fragments of anti- β_2m antibodies. The fragments were shown to interfere with the recognition phase of the MLR by selectively affecting the responder T lymphocytes and not the stimulator cells. These data might

be interpreted as if β_2m constitutes a subunit of lymphocyte surface structures of importance to the recognition phase. In experiments with cell-mediated lympholysis, however, Lightbody et al. (68) could not find that anti- β_2m antibodies affect the cytotoxicity of the effector cells. Nor did preincubation of the target cells with anti- β_2m protect these cells from lysis.

The lymphocyte transformation induced by purified protein derivative (PPD) is impeded by anti- β_2m antibodies (3). Such antibodies also seem to reduce the response to "T-cell mitogens" (PHA, Con A) whereas no effect was detected on the response to the two "B-cell mitogens" LPS and D x S (cf.ref. 72).

β_2m in pathological conditions

This subject has recently been authoritatively reviewed (52) and the following represents only a summary of the most prominent and well-established findings. From numerous investigations about the diagnostic and prognostic significance of determining β_2m in body fluids during various pathological conditions, three main groups of diseases can be distinguished; renal disorders, inflammatory diseases and malignant diseases.

β_2m was originally isolated from the urine of patients with renal tubular dysfunction, because such urine was found to contain large amounts of the protein (7). This is explained by the fact that β_2m and other low molecular weight plasma proteins readily pass the glomerular membrane into the primary filtrate. During normal conditions the filtrated proteins are reabsorbed in the cells of the proximal tubules (9,94) where they are degraded to amino acids (76). Thus renal disorders afflicting the proximal tubules (Fanconi syndrome, cadmium intoxication, acute tubular necrosis of

various aetiology, etc.) will cause an increased urinary excretion of $\beta_2\text{m}$. This means that the output of $\beta_2\text{m}$ is a useful and sensitive marker of the proximal tubular function. Plasma levels of $\beta_2\text{m}$ are dependent on the glomerular filtration rate. A reduction of this rate (seen for instance in glomerular and circulatory disorders) reduces the catabolism of the protein and causes high plasma levels. This must be borne in mind when discussing the level of $\beta_2\text{m}$ in plasma during supposed non-renal pathological conditions.

High plasma levels of $\beta_2\text{m}$ have been noted in patients with auto-immune diseases such as rheumatoid arthritis and systemic lupus erythematosus (74) and auto-antibodies to $\beta_2\text{m}$ have been detected in sera of patients suffering from the latter disease (105). Increased plasma $\beta_2\text{m}$ values have been demonstrated among patients with various neoplastic diseases (for references see review, ref. 52). Especially tumours originating from lymphoid cells seem to be followed by high $\beta_2\text{m}$ levels. Analysis of plasma $\beta_2\text{m}$ in these neoplastic conditions seem to be of both diagnostic and prognostic potential, whereas the significance in many other malignant diseases appears more doubtful. The mechanisms behind the elevation of plasma $\beta_2\text{m}$ in chronic inflammatory and malignant diseases are not clear. It could perhaps, in the former case, be explained by activation of inflammatory and lymphoid cells. In malignant disorders, the $\beta_2\text{m}$ level has been reported to be correlated to the total tumour mass (117) which may indicate that elevated $\beta_2\text{m}$ merely reflects the increased cell turn over and general catabolic condition seen in malignant disease. Another and perhaps more interesting possibility, is that the elevation of $\beta_2\text{m}$ depends on an activation of the immune system secondary to the malignant process.

Concluding remarks

The function of β_2m is still unknown. The main scientific interest in this protein is based on its multiple relations to important molecules of the immune system, such as immunoglobulins and gene products of the major histocompatibility complex. Further study of β_2m promises to contribute to a deepened knowledge of how the immune system functions and, reversely, it also seems plausible that increased knowledge of this complex system will make it easier to understand the biological role of β_2m .

PRESENT INVESTIGATION

Purification and some properties of rabbit β_2m (I)

The first animal homologue of human β_2m to be isolated was dog β_2m (112). This study used urine from kidney transplanted dogs as starting material. The dog homologue was followed by rabbit β_2m in 1974, when Ingemar Berggård published a preliminary report on the isolation and some properties of this protein (8). In this work, tubular proteinuria was induced with sodium chromate, a substance known to be toxic to the cells of the proximal tubules of the rat kidney and capable of inducing lysozymuria in these animals (27). Compared with cadmium (98) and maleic acid (Ingemar Berggård and Barbro Berggård, unpublished), which are both known to be nephrotoxic, sodium chromate was found to be a more efficient inducer of tubular proteinuria in the rabbit. Its general toxic effect is also less prominent and in the doses used in the original (8) and the present study (I), the rabbits did not seem to suffer and mortality was low. The urinary excretion of β_2m was increased about 100-fold with a maximum two days after administration of sodium chromate. In our laboratory this nephrotoxic agent was successfully used to cause low molecular weight proteinuria also in guinea pigs (17), rats (71) and mice (Bo Åkerström and Ingemar Berggård, unpublished), thereby providing good starting materials for the isolation of β_2m from these species.

Rabbit β_2m was purified by ultrafiltration, gel chromatography, zone electrophoresis and ion-exchange chromatography, which is the same protocol as previously published (8). The protein was measured by single radial immunodiffusion during the purification and yields were satisfactory (19-26%). On

zone electrophoresis, β_2m appeared in two peaks. β_2m with abnormal electrophoretic mobility has previously been described in human (46), guinea pig (17) and rat urine (71). Previous investigations of human β_2m samples (102,122) and the immunoelectrophoretic analysis of rabbit sera in the present study failed to reveal any genetic polymorphism of β_2m . Our data rather support the idea that the abnormal β_2m represents β_2m that was degraded in the kidney or in urine. The results, however, do not exclude the possibility that both forms occur in blood, nor the possibility of a genetic polymorphism as the basis for the observed charge heterogeneity. The minor form could be present in concentrations below the detection limit of our assay and genetic polymorphism of β_2m has recently been detected in mouse (79,106).

The complete amino acid sequence of rabbit β_2m has been published, and comparison of this sequence with that reported from human β_2m showed a homology of 71% (38). Our data on some physicochemical properties of the rabbit protein are purely descriptive, but emphasize the close resemblance between various β_2m homologues (for a comparison with human β_2m , see Table 1, page 9).

In rabbit serum, β_2m was found in at least two high-molecular-weight complexes. The larger of these was eluted closely before, and the smaller just after the albumin peak on Sephadex G-200. The latter material has a K_{av} identical to papain-solubilized H-2 antigens (58) and perhaps represents shed RLA antigens. RLA and HLA antigens have a tendency to aggregate (54, 123), and the larger complex could perhaps represent RLA dimers, whereas the peak, occasionally seen at the void volume, could harbour larger such aggregates. In mouse serum, β_2m is found in an additional 300-400,000 dal-

ton complex (60,84). Apart from β_2m , this complex contains polypeptides with molecular weights of 42,000, 65,000 and 75,000 (60). The 42,000 subunit seems to be the one associated with β_2m . It does not carry H-2 allo-antigenic activity (60,84) but reacts with heteroantisera against H-2 antigens (60,85). The mouse serum complex appears unstable (60), which could explain why the corresponding component is not detected in rabbit serum. If the complex is actually degraded, this could mean that the β_2m -containing material eluted closely after albumin is comprised of rabbit equivalents to the above-mentioned H-2-like 42,000 dalton polypeptides. This notion is perhaps supported by our finding that mouse and horse sera contain unstable material (in the K_{av} range 0,08-0,15 on Sephadex G-200) with affinity for exogenously added, heterologous β_2m (73). If, however, antigenic β_2m determinants are hidden in these high-molecular-weight complexes, this could be an alternative explanation to the fact that the material is not detected in rabbit (I), rat (71), or guinea pig serum (73) by homologous RIA.

β_2m in mammalian lymphocytes and erythrocytes. Presence and association with MHC gene products (II,III)

In both mouse and rat, classical transplantation antigens (H-2K, D, L and AgB antigens) are expressed on erythrocytes, whereas the corresponding antigens in rabbit (RLA), guinea pig (GPLA), and humans (HLA-A, -B, and -C) are usually not present on red blood cells. Investigations performed with the EB virus transformed Daudi cell line have demonstrated an independent expression of β_2m and the heavy HLA chain (92). Studying erythrocytes from various mammalian species offered a possibility to analyse the relation between the two transplantation antigen subunits on non-malignant cells. The results (II) indicate that on such cells these subunits are expressed together. Paper II also contains some quantitative data on the

number of β_2m molecules in lymphocytes of four mammalian species. The values suggest a similar β_2m density among the cells.

One of the most significant discoveries in immunology during the past decade was the demonstration that β_2m represents the light HLA chain (44,81,96). When we started to investigate RLA and GPLA antigens this information was new, and our work intended to see whether the same principal structure was to be found also in species other than man. During the course of the investigation, the subunit structure of H-2K and D antigens was established (82,104,110). This made our own observation (III) that RLA and GPLA antigens in fact also have a β_2m subunit rather confirmatory, but it could contribute to a more generalised picture of the structure of these MHC gene products.

In 1975, β_2m was reported to be a structural feature of allogeneic effect factor (AEF) (2). Antisera against immune response region associated (Ia) antigens block both AEF and MLR, which made it reasonable to speculate that β_2m was to be found also in association with Ia antigens. Indeed, Callahan et al. (15) found that Ia antigens isolated from high density lipoproteins of normal mouse serum were retained on anti- β_2m antibody immunosorbents. Bound antigens were also capable of reacting with anti-Ia antiserum. Our observation in the guinea pig (III), and evidence from several other laboratories on the structure of Ia antigens in mouse (19, 22,125) did not subscribe to these observations. The reason for this discrepancy is not clear. Most present data, however, do suggest that Ia antigens lack a β_2m subunit, although it is too early to exclude the possibility that β_2m is associated with other gene products of the I-region.

Some evolutionary aspects on β_2m (I, IV)

The evolutionary approach to the study of a molecule of unknown function could contribute to the understanding of its nature and significance. The multiple relations between β_2m and molecules of the immune system give evolutionary investigations of β_2m an extra potential.

Analysis of the primary structure of the myeloma protein Eu (24) provided evidence for the hypothesis (48,111) that immunoglobulins evolved by the duplication of an ancestral gene coding for a polypeptide chain of 100-110 amino acids. These ideas were further developed when it was suggested that immunoglobulin genes have evolved from genes specifying histocompatibility antigens (12,37). The close resemblance between β_2m and immunoglobulin domains (95,113) points towards a common precursor gene for these molecules. Moreover, as β_2m is associated with the heavy chain of the major histocompatibility antigens (44,81,96) and as this latter polypeptide is also structurally related to immunoglobulins (90,97,121) the idea is supported of a common evolutionary origin of immunoglobulins, classical transplantation antigens, and β_2m . Vertebrates are the only animals known to produce immunoglobulins. A demonstration that a β_2m -like molecule is present and associated with a polypeptide homologous to the heavy chain of vertebrate transplantation antigens, also in invertebrates, would add further support to this hypothesis.

Immunological cross-reactivity has been shown between β_2m of various mammalian species (8,17,71) and also between human β_2m and the chicken homologue (61). Paper I showed that antibodies against rabbit β_2m were cytotoxic to various mammalian and chicken lymphocytes and precipitated guinea pig and rat β_2m . In lymphocytotoxicity and hemagglutination experiments, we tried to detect β_2m -homologues also among lower species. Natural-

ly occurring cytotoxins and agglutinins complicated this study. Despite big efforts our only progress was a weak, but specific, agglutination of erythrocytes from the aquarium fish Puntius Leptus. This positive result was obtained with goat anti-rabbit β_2m IgG antibodies used in the indirect (Coombs) haemagglutination test (L. Björck and I. Berggård, unpublished). β_2m -like activity has also been detected in the serum of sand sharks utilising a heterologous RIA based on the cross-reactivity between rabbit and human β_2m (40).

Results obtained with cellular techniques, such as complement dependent cytotoxicity, immunofluorescence, and agglutination procedures, are often difficult to interpret because of unspecific antibody reactions. Paper IV describes another assay based on the assumption that the interaction between β_2m and the heavy transplantation antigen chain engages structures that have been conserved during evolution. Therefore, papain solubilized AgB antigens were purified from WF and BN rats. The isolation procedure included ion-exchange chromatography, affinity chromatography on Lens culinaris-Sepharose 4B and gel filtration. Thus purified antigens could specifically bind not only rat β_2m but also β_2m isolated from other mammalian species. Inhibition of this interaction between the heavy AgB chain and heterologous β_2m was used to detect β_2m -like molecules in fish sera (IV). With this assay, a battery of anti-body-based heterologous RIAs, and the heterologous interaction between cod splen cells and mammalian β_2m , we have recently identified β_2m -like molecules also in extracts of two invertebrate species; the crayfish (Cambarus diogenes) and the earthworm (Lumbricus terrestris) (Shalev, A., Greenberg, A.H., Lögdberg, L. and Björck, L. Manus submitted for publication).

Immunogenicity and evolutionary conservatism of a protein are inversely correlated (89). Although rabbits and humans, for instance, are evolutionarily closely related, it is still possible to raise antisera against human β_2m in rabbits. Moreover, the above-mentioned invertebrate extracts, often inhibited the various assays surprisingly efficiently if one considers that amino acid sequence comparison between human and rabbit β_2m has demonstrated as much as 29 per cent sequence difference (20,38). There is perhaps a discrepancy between these data and the fact that β_2m also appears well conserved during evolution. On the other hand, it is possible to explain these opposite evolutionary trends if there has been a rapid diversification of the β_2m gene (s) at various stages of evolution, including mammalian. If correct, this suggests that invertebrate β_2m could be as homologous to mammalian β_2m s as these are to each other. This speculation, however, before being established as truth, must await the isolation and structural characterization of β_2m of lower species.

Immunosuppressive effect of anti- β_2m antibodies (V)

Antibodies against β_2m were found to prolong the survival of heart allografts in rats (V). The immunosuppressive effect was weak, which was emphasized both by the short prolongation time and by the large doses required. From a theoretical point of view, however, this moderate, but significant, effect of anti- β_2m antibodies could be of interest.

As the mechanisms behind the immunosuppression are still unclear, one can only speculate on how the antibodies interfere with the immune response. One possibility is that they simply kill a number of immunocompetent cells by complement dependent cytotoxicity. Antibodies against β_2m have been reported to inhibit the MLR (3,70) by impeding the responding cells (70). They also seem to block lymphocyte transformation induced by antigens and

mitogens (for references see ref. 5). β_2m is part of major histocompatibility antigens which are target molecules for cytotoxic T-lymphocytes (see refs 23,55). These data indicate that β_2m could be part of receptor structures on T-lymphocytes, and the structure(s) that T-killer cells have to recognize on various target cells (including for instance cells of transplanted heart allografts) before effectuating their killing function. If true, anti- β_2m antibodies could react with such structures, thereby causing the in vivo immunosuppression described in paper V. Anti- β_2m antibodies are capable of inducing lymphocyte transformation (for references see ref. 5). If preferentially T-suppressor cells are activated, this could also impede the immune response. Theoretically this could be accomplished also if the antibodies cross-react with the T-lymphocyte antigen receptor, which seems to be an immunoglobulin-like molecule (11). Moreover, one can speculate on the possibility that the immunosuppressive properties of anti- β_2m antibodies are caused by a reaction between these antibodies and cell-to-cell interaction factors. Thus association of β_2m with such factors has been reported (2,103).

Interaction between β_2m and bacteria (VI, VII)

β_2m is structurally related to immunoglobulins (95,113) which have affinity for protein A of staphylococci (36), and cell surface structures of many streptococcal strains (56). This was the knowledge that stimulated us to investigate whether also β_2m was able interacting with Fc-reactive bacteria. Our early studies used monomeric, radiolabelled β_2m in uptake experiments which proved completely negative. The lack of reactivity between monomeric β_2m and staphylococcal protein A was also reported by Ochi et al. (88). In the work by Peterson et al. (97) H-2 antigens were found to bind to protein A, and this binding was more pronounced if the antigens were aggregated. Thus, in order to increase the avidity in a pos-

sible reaction between β_2m and bacteria, the protein was aggregated and again tested in binding assays with various bacterial strains. All group A and some group C and G streptococcal strains showed uptake of radiolabelled, aggregated human β_2m , whereas five other gram-positive and three gram-negative bacterial species did not (VI). Some group B strains were slightly positive, and subsequent work has demonstrated that these strains also possess a β_2m -binding with many characteristics in common with the interaction between β_2m and A, C, and G streptococci (109). Moreover, many oral streptococcal strains belonging to Streptococcus mutans, sanguis, mitior, salivarius, and milleri also bind β_2m (25,26).

Somewhat puzzling, it was realised that the β_2m -binding structures on streptococci seemed to be different from those responsible for Fc-binding (VI). Thus β_2m and IgG-binding were not correlated among the various tested A, C and G strains. Moreover, β_2m -uptake was trypsin-sensitive, whereas IgG reactivity was not. Finally binding of β_2m was not inhibited by IgG. The fact that the bacterial "receptor" for β_2m seems distinct from "receptors" for immunoglobulin, differs from what has been reported for S. Mansoni shistosomula. These micro-organisms appear to bind both immunoglobulin and β_2m to the same "receptor" (120). The lacking correlation between β_2m and IgG uptake is also in contrast to the almost identical patterns obtained when 72 strains of A, C, and G streptococci were tested for uptake of both β_2m and fibrinogen (57). Furthermore, the binding of β_2m was inhibited by fibrinogen (57, VII).

In 1928, Rebecca Lancefield described antiphagocytic surface antigens, M proteins, on group A streptococci, which are of primary importance to the virulence of these micro-organisms (63,65). Thus type-specific opsonic antibodies against M proteins, neutralizing their antiphagocytic effect, are

necessary for the development of resistance to streptococcal infections (65,66). M proteins are highly elongated proteins with molecular weights of 30,000-35,000, and they are extremely heat-stable (4,34,63). This latter property also characterizes the streptococcal " β_2m receptor". Thus, if β_2m -binding bacteria are heated at 95°C for five minutes, this does not affect their binding capacity (Björck, L. and Kronvall, G., unpublished). M proteins are, similar to the β_2m -binding structures, trypsin-sensitive (64, VI). As mentioned above, fibrinogen and β_2m -binding to A, C, and G streptococci are closely related. M proteins are known to interact with fibrinogen (50,119). M protein-carrying group A streptococcal strains were found to bind β_2m , whereas M protein-negative variants of these strains did not (VII). These different data all suggest that the binding of aggregated β_2m to group A streptococci is mediated by M proteins, and in group B, C, and G streptococci by M protein-like molecules.

The biological and pathological significance of the interaction between β_2m and streptococci is far from clear. As aggregates of β_2m are required, a multipoint attachment seems to be necessary for β_2m -binding to take place. Such an attachment could be created between cell-bound β_2m molecules and adhering bacteria. Thus the adherence to epithelial cells of β_2m -binding bacteria is partly inhibited if the cells are pretreated with antibody fragments directed against β_2m (Björck, L., Söderström, M. and Kronvall, G., unpublished). Experiments of this kind must be interpreted cautiously, as unspecific alterations in the cell membrane, after the reaction between the antibodies and β_2m , are difficult to exclude. Despite this reservation, the results indicate that there actually is an interaction between cell-bound β_2m and structures of the streptococcal cell surface, presumably M proteins. In the host-parasite relation such an interaction appears beneficial for the parasite if the cells are of epithelial

origin. In this case, cell-bound β_2m could act as "landing-gears" for infecting streptococci. If, on the other hand, the bacteria adhere to lymphocytes and/or phagocytic cells, this could be favourable to the host. Apart from a possible role for β_2m in the complicated host-parasite relation, the interaction between a mammalian protein, intimately related to the immune system, and bacteria appears to be of interest also from a more general biological point of view. To gain further and deeper knowledge about this interaction requires the chemical isolation and characterization of relevant β_2m -binding bacterial surface structures.

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APPENDIX

PAPERS I - VII

I

STUDIES ON β_2 -MICROGLOBULIN IN THE RABBIT

by

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ABSTRACT

β_2 -Microglobulin has been isolated from urine of rabbits treated with sodium chromate. The purification schedule included ultra-filtration, gel chromatography, zone electrophoresis and ion-exchange chromatography. During the isolation the protein appeared in two forms with different electrophoretic mobility. In rabbit sera, however, no evidence was obtained suggesting that free β_2 -microglobulin occurs in such different forms. Physicochemical characterisation of the major type revealed a molecular weight of about 11,500, a sedimentation coefficient of 1.65 S, a Stokes' radius of 1.6 nm, and an isoelectric point at 5.9. The molecule has a low frictional ratio (1.11) suggesting a compact, globular shape. Antibodies against rabbit β_2 -microglobulin were found to be cytotoxic not only to rabbit and other mammalian lymphocytes but also to chicken lymphocytes. In rabbit serum β_2 -microglobulin was found in free form and in at least two different high molecular weight complexes. In splenocyte lysates a β_2 -microglobulin-containing molecule with the same size as the larger of the two complexes was identified.

INTRODUCTION

β_2 -Microglobulin (β_{2m}) was originally isolated from human urine but is also found in other body fluids (Berggård and Bearn, 1968). The protein is produced by various types of cells (Nilsson et al., 1973) and occurs bound to cell surfaces (Peterson et al., 1972; Poulik, 1973). Structurally β_{2m} is closely related to immunoglobulins (Smithies and Poulik, 1972; Peterson et al., 1972), and it has been shown to represent the light chain of major histocompatibility antigens (Grey et al., 1973; Nakamuro et al., 1973; Peterson et al., 1974). Homologues of β_{2m} have been isolated from dog (Smithies and Poulik, 1972), rabbit (Berggård, 1974), mouse (Natori et al., 1975), rat (Callahan et al., 1976; Lögdberg et al., 1979), guinea pig (Cigén et al., 1978), cow (Groves and Greenberg, 1977) and chicken (Winkler and Sanders, 1977). This has simplified structural, evolutionary and functional studies of the protein. In the mouse, β_{2m} is found associated not only with the major H-2 histocompatibility antigens but also with the closely related thymus leukemia (TL) and Qa-2 antigens (Östberg et al., 1975; Vitetta et al., 1975; Michaelson et al., 1977). Antibodies against β_{2m} have been reported to interfere with the cellular response in various immunological in vitro reactions (for references see Berggård et al., 1980) and in vivo anti- β_{2m} prolong the survival of rat heart allografts (Björck et al., 1977).

In clinical medicine determination of β_{2m} in plasma and other body fluids has been used as a diagnostic tool. For instance, increased plasma levels of β_{2m} is a sensitive parameter of renal failure (Bernier et al., 1968; Peterson et al., 1969) and certain neoplastic and autoimmune conditions are also followed by elevated plasma β_{2m} (Evrin et al., 1973).

In the present work the isolation and some physicochemical characteristics of rabbit β_{2m} are described. Its relations to other β_{2m} s were investigated in immunochemical and lymphocytotoxicity experiments and the occur-

rence of β_2m and β_2m -containing molecules was studied in rabbit sera and splenocyte lysates. Some preliminary data on the purification of rabbit β_2m have previously been reported (Berggård, 1974) and the amino acid sequence of the protein has been determined (Gates et al., 1979; Cunningham and Berggård, 1975; Poulik and Smithies, 1979).

MATERIALS AND METHODS

Urine

Outbred rabbits were given single injections of sodium chromate (10 mg/kg of body weight of a solution containing 20 mg/ml) subcutaneously and five 24-h urine specimens were collected.

β_2 -Microglobulins and antisera

Human β_2m (Berggård and Bearn, 1968), guinea pig β_2m (Cigén et al., 1978) and rat β_2m (Lögdberg et al., 1979) were purified in this laboratory. Antisera were raised in three goats against purified rabbit β_2m as previously described (Björck et al., 1977).

Lymphocytes

Purified lymphocytes were prepared from rabbit, guinea pig, human, rat and chicken blood by gradient centrifugation (Böyum, 1968) on Lymphoprep, density 1.077 (Nyegaard and Co., Oslo, Norway).

Other materials

DEAE-cellulose 23-SH was obtained from Serva Feinbiochemica, Heidelberg, Germany. Pevicon C-870 was from Kema Nord AB, Stockholm, Sweden. Carrier ampholytes (Ampholine) were from LKB-Produkter AB, Bromma, Sweden, and 23/32-in. dialysis tubing was from Union Carbide Corp., Chicago, Ill., USA. Sephadex G-100 and G-200 and Sepharose 4 B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Lens culinaris haemagglutinin was isolated as previously described (Hayman and Crumpton, 1972) and coupled to Sepharose 4 B (Cuatrecasas, 1970). Alpha-methylmannoside was from Sig-

ma Chemical Co., St. Louis, Mo., USA. 125 I was a product of Amersham, Buckinghamshire, England. All other chemicals were reagent grade or of the best quality available.

Protein determinations

Protein concentrations were measured by the modified Folin method of Lowry et al. (1951), or by reading the absorbance at 280 nm. In the Folin method human IgG was used as standard.

Concentrations of proteins

Urine and protein solutions were concentrated by ultrafiltration (Berggård, 1961). Urine was concentrated 60- to 100-fold and centrifuged before purification of β_2 m.

Serum samples tested on immunoelectrophoresis were concentrated in the following way: 15 ml of serum was diluted twice with phosphate buffered saline, pH 7.4, and filtrated through an XM 50 membrane (Amincon, Lexington, Mass., USA) at a pressure of 3×10^5 Pa. The filtrate containing low molecular weight serum proteins, was then concentrated to about 100 μ l by ultrafiltration with a 23/32 in. dialysis tubing, Ten μ l of this material was finally subjected to immunoelectrophoresis.

Electrophoretic methods and isoelectric focusing

Preparative zone electrophoresis in blocks of Pevicon C-870 (Berggård and Bearn 1968) was performed in 0,1 M borate buffer, pH 8,9. Polyacrylamide gel electrophoresis in slabs with sodium dodecylsulphate and isoelectric focusing was carried out as described (Neville, 1971, Cigén et al., 1978).

Immunochemical techniques

Immunodiffusion in gel according to Ouchterlony, immunoelectrophoresis and single radial immunodiffusion were performed as previously reported (Berggård and Bearn, 1968). Radioimmunoassay of β_2 m was carried out u-

sing the polyethylenglycol-exclusion method (PEG 6000, Kebo AB, Stockholm, Sweden) described by Plesner et al., (1975). β_2m was ^{125}I -labeled by the Chloramin-T method (Greenwood et al., 1963). Inhibition curves were made by plotting counts/min against the log of the concentration. The detection limit of the radioimmunoassay was never above 1 $\mu g/l$.

Ultracentrifugation, estimation of Stokes' radius

Sedimentation velocity and equilibrium experiments and determination of Stokes radius (r_s) was performed as previously reported (Cig n et al., 1978 and Karlsson et al., 1972).

Calculations of the apparent diffusion coefficient of the molecular weight by the Svedberg equation and of the frictional ratio

The apparent diffusion coefficient ($D_{20,w}$) was calculated using Stokes-Einstein's equation (Gosting, 1956). The molecular weight was determined from the diffusion coefficient and the sedimentation coefficient by Svedberg's equation. The frictional ratio (f/f_0) was computed from the sedimentation coefficient and the molecular weight derived from the amino acid composition of the protein. A partial specific volume for the calculations was estimated from the amino acid composition as described by Cohn and Edsall (1943). The formulas used have been reported by Svedberg and Pedersen (1940).

Lymphocytotoxicity tests

All studies were performed with a two-step microlymphocytotoxicity technique (Terasaki and McClelland, 1964). The specificity of the reactions was investigated by adding purified rabbit β_2m to the antisera to final concentrations ranging from 10 to 100 $\mu g/ml$. After incubation at 20°C for 2 h and at 4°C overnight the absorbed antisera were used for cytotoxicity tests.

Solubilisation of splenocyte membrane proteins

Rabbit spleens were homogenised in 0.05 M TRIS-HCl buffer, pH 7.5, containing 0.25 M sucrose, 0.025 M KCl and 0.005 M $MgCl_2$ using an Omnimixer 17220 (Sorvall Inc., Conn., USA). The homogenate was centrifuged at $10,000 \times g$ for 10 min. The supernatant was subjected to a second centrifugation at $105,000 \times g$ for 45 min. The resulting pellet was Potter-homogenised in 0.05 M TRIS-HCl buffer, pH 8.6, containing 0.5% Na-deoxycholate (DOC) and 0.15 M NaCl. Incubation for 30 min at $4^\circ C$ was followed by centrifugation at $105,000 \times g$ for 45 min. The supernatant was finally used in gel chromatography experiments.

RESULTS

Purification of rabbit β_2 -microglobulin

Urine from apparently healthy rabbits contain only small amounts of β_2m (mean value among six rabbits: 0.7 mg/l, range 0.2-0.9 mg/l) and the daily excretion is consequently low (mean value among six rabbits: 0.14 mg/day, range 0.09-0.25 mg/day). As demonstrated in Fig. 1 sodium chromate increases the urinary excretion with a factor of about 100 two days after administration of the nephrotoxic agent, when a maximum is reached. Urine from sodium chromate-treated rabbits was concentrated and used as starting material. The following isolation steps included gel chromatography on Sephadex G-100, preparative zone electrophoresis in borate buffer and ion-exchange chromatography on DEAE-cellulose. In Fig. 2 representative graphs have been selected from different preparations. A purification protocol is summarised in Table 1. The yield varied between 19 and 26 % of the total amount of β_2m found in concentrates of urine. Antisera were raised against the first preparations of rabbit β_2m and were used to trace and measure the protein during the following purifications. As shown in Fig. 2b β_2m appeared in two peaks on zone electrophoresis. The quanti-

tatively dominating cathodal material was further purified on DEAE-cellulose.

Purity and apparent size homogeneity

The purity of the isolated protein was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Only single protein bands were seen. The results of isoelectric focusing experiments also indicated purity (see below).

Physicochemical properties

Some physicochemical properties of rabbit β_2m are presented in Table 2. All figures are average values from analyses of at least two different preparations. The molecular weights obtained by calculations from the sedimentation and diffusion coefficients, from sedimentation equilibrium data or from the amino acid composition indicate a value of about 11,500. On polyacrylamide gel electrophoresis in sodium dodecyl sulfate the mobility of rabbit β_2m was indistinguishable from that of human $\beta_2 m$. The absorption coefficient at 280 nm and pH 7.0 was estimated to be $1.61 \times 10^4 M^{-1} cm^{-1}$ with a value of 11,500 for the molecular weight. Isoelectric focusing experiments gave single, symmetrical peaks with the pI values given in Table 2.

Immunological cross-reactions

On Ouchterlony immunodiffusion analyses, antisera against rabbit β_2m precipitated not only rabbit β_2m but also the guinea pig and the rat β_2ms . The two latter proteins gave reactions of partial identity with rabbit β_2m . Figure 3 illustrates the immunological cross-reaction between the guinea pig and rabbit proteins.

In lymphocytotoxicity experiments, the three goat antisera against rabbit β_2m used in this study were clearly cytotoxic not only to rabbit lymphocytes but also to other mammalian lymphocytes (human, guinea pig and rat lymphocytes were examined) and to chicken lymphocytes. The cyto-

toxic activity against heterologous lymphocytes most often was somewhat lower than the activity against rabbit lymphocytes. Non-immune goat serum did not have significant toxicity to any of the lymphocytes examined. The specificity of the lymphocytotoxicity was evaluated by absorptions of the antisera with purified rabbit β_2m . Lysis of lymphocytes from all species was decreased to background levels after such absorptions. The results of these experiments indicate that all lymphocytes examined carry β_2m on their surfaces and that the various β_2ms cross-react immunologically.

β_2 -microglobulin in rabbit serum and splenocyte lysates

The content of β_2m in rabbit serum was measured with radioimmunoassay. Nine individual rabbit sera from apparently healthy animals were analysed and gave a mean value of 3.2 mg/l, range 2.6-4.5 mg/l. Sera from several rabbits were separated on a Sephadex G-200 column and the effluent was analysed for β_2m with radioimmunoassay (Fig. 4a). Three β_2m -containing peaks were always seen. In two of the sera the first eluted β_2m peak was divided into two clearly separated peaks. Serum was also chromatographed in the presence of 0.5% DOC and the same principal elution profile was obtained. Among splenocyte membrane proteins, solubilised with DOC, only one high-molecular weight β_2m complex was detected (see Fig. 4b). Part of this DOC-solubilised material was also submitted to affinity chromatography on Sepharose 4B-coupled Lens culinaris haemagglutinin. The column was eluted with 10% α -methylmannoside. Glycoproteins thus prepared appeared as a single peak on Sephadex G-200 at the same position as the high-molecular weight peak in Fig. 4b, suggesting that this peak includes glycoproteins with affinity for Lens culinaris.

Immunoelectrophoretic analysis of β_2 -microglobulin in rabbit sera

After concentration of the low molecular weight proteins in rabbit sera (see above), it was possible to achieve visible precipitates between se-

rum $\beta_2\text{m}$ and anti-rabbit $\beta_2\text{m}$ antibodies in immunoelectrophoretic experiments. Serum from 80 different rabbits were examined in order to investigate if any polymorphism could be detected in this population. No such evidence was found since $\beta_2\text{m}$ had the same mobility in all sera analysed. It was also noticed that $\beta_2\text{m}$ in these serum samples had the same mobility as $\beta_2\text{m}$ from the major zone electrophoresis peak (see Fig. 2b) whereas material from the minor peak migrated somewhat faster.

DISCUSSION

Low molecular weight proteins like $\beta_2\text{m}$ are eliminated from the blood via glomerular filtration followed by tubular reabsorption and catabolism. $\beta_2\text{m}$ was originally isolated from human urine obtained from patients with renal tubular disease (Berggård and Bearn, 1968). In healthy animals the urinary excretion of $\beta_2\text{m}$ is low. As described above, sodium chromate greatly increases this excretion, probably by inducing renal tubular damage (Evan and Dail, 1974). In the present investigation urine from rabbits treated with this nephrotoxic agent was used as starting material.

During the purification of rabbit $\beta_2\text{m}$ two forms with different electrophoretic mobility were seen. Two variants of human $\beta_2\text{m}$ differing in isoelectric point have previously been described (Hall *et al.*, 1977) and also during the isolation of guinea pig and rat $\beta_2\text{m}$, variants of the protein were observed (Cigén *et al.*, 1978; Lögdberg *et al.*, 1979). All these $\beta_2\text{m}$ homologues were isolated from urine. Since genetic variants of $\beta_2\text{m}$ from any species could be valuable in determining its biological function, the low molecular weight serum proteins from 80 rabbits were analysed with immunoelectrophoresis in search of such a variant. In all sera $\beta_2\text{m}$ showed the same mobility thereby providing no evidence for the presence of any $\beta_2\text{m}$ variant in rabbit serum. Perhaps do the different forms of the protein seen

in urine arise by degradation of native β_2m in the kidney or in urine, for instance by proteolysis or deamidation. The fact that the electrophoretic mobility of β_2m in the serum samples was identical to the mobility of the major form of the protein in urine, whereas the minor form migrates somewhat faster, supports this opinion.

In the present study it was established by immunodiffusion experiments that anti-rabbit β_2m reacts not only with rabbit β_2m but also with β_2m isolated from other mammalian species, and in cytotoxicity experiments the antibodies were found to cause lysis of mammalian and chicken lymphocytes. Gordon and Kindt (1976) have reported evidence for cross-reactions between β_2m from several vertebrate species. They found that sera from these species inhibited heterologous radioimmunoassays based on the cross-reaction between rabbit and human β_2m . These data and those presented here indicate that β_2m has been conserved during evolution. The structural similarity between β_2m , immunoglobulin domains and major histocompatibility antigens (Peterson et al., 1975; Terhorst et al., 1977; Trägårdh et al., 1978) has provided support for the hypothesis (Gally and Edelman, 1972; Bodmer, 1972) that the immunoglobulin genes have evolved from genes specifying histocompatibility antigens. A demonstration that β_2m is present not only in vertebrates, which are the only animals known to produce immunoglobulins, but also in invertebrates would add further support to this interesting theory.

More than 50% of serum β_2m as measured by radioimmunoassay was found in two high molecular weight forms under the third protein peak given by serum separated on Sephadex G-200. The larger of these β_2m complexes has the same size as the β_2m -containing molecules released from rabbit spleen cells with detergent (see Fig. 4) and may represent rabbit major histo-

compatibility antigens (RLA), molecules which have been isolated by the use of antirabbit β_2m affinity columns (Kimball et al., 1979). The second β_2m peak has a K_{av} value (0.43) almost identical to what has been reported for papain-solubilised H-2 antigens (Kvist and Peterson, 1978). The two forms of β_2m complexes could both be shedded from cells to serum, and the fact that the peak corresponding to the larger complex sometimes was split into two, might indicate that there is a gradual degradation of the larger complex occurring in serum. In one of the sera analysed, a small β_2m peak was also detected at the void volume and serum might contain additional β_2m material not measured here because of low reactivity with anti-rabbit β_2m . Thus, in rabbit serum we have not been able to detect the β_2m -containing protein with a molecular weight of about 300 000 - 400 000 d that has been described in mouse serum (Natori et al., 1976; Kvist and Peterson, 1978).

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Table 1. Purification of rabbit β_2 -microglobulin.

The urinary proteins were obtained by collecting 24-hour specimens of urine from 6 rabbits. Total protein was determined with the Folin method in the two first steps, and in the two following steps from the optical density at 280 nm and by weighing the protein. β_2 m was measured by single radial immunodiffusion.

Purification step	Total protein (mg)	β_2 -microglobulin (mg)	Yield (%)	Purity (%)
Concentrated urinary protein	2777	262	100	9
Sephadex G-100 chromatography	276	156	60	57
Zone electrophoresis	84	82	31	98
DEAE-cellulose chromatography	67	67	26	100

Table 2. Physicochemical properties of rabbit $\beta_2\text{m}$

Sedimentation coefficient, $s_{20,w}^0$	1.65 S
Stokes' molecular radius, r_s	1.6 nm
Apparent diffusion coefficient, $D_{20,w}$	137 $\mu\text{m}^2/\text{s}$
Partial specific volume, V (calculated) ^a	0.732 ml/g
Molecular weight	
From sedimentation and diffusion coefficients	10,900
By sedimentation equilibrium ultracentrifugation	11,000
From amino acid composition	11,655
Frictional ratio, f/f_0	1.11
Absorption coefficient at 280 nm (pH 7.0)	$1.61 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$
Isoelectric point at 5°C	6.0
at 20°C	5.9

^a From amino acid composition (Berggård, 1974; Gates et al., 1979)

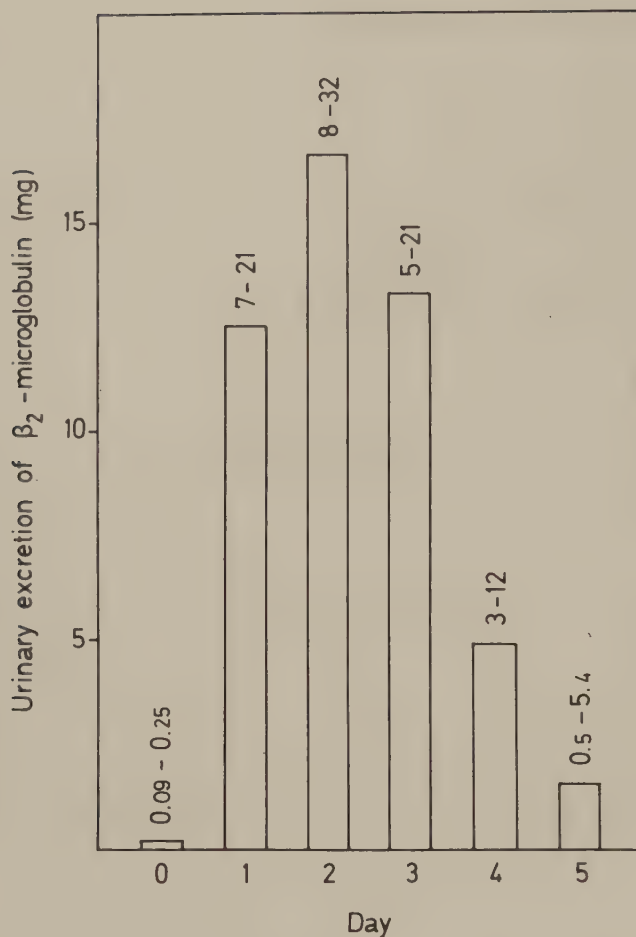


Fig. 1. The effect of sodium chromate on the urinary excretion of β_2 m. Six rabbits were given 10 mg/kg body weight subcutaneously at the end of day 0 and individual 24 h urine specimens were collected. β_2 m was determined by radioimmunoassay. Columns represent mean values among the 24 h specimens and ranges are given.

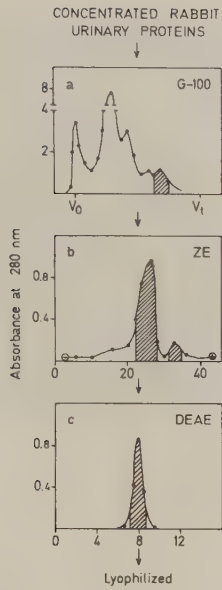


Fig. 2. Purification of rabbit $\beta_2\text{m}$. The graphs show protein distribution plotted against elution volume (a), fraction number (b) or conductivity ($\text{m } \Omega^{-1}\text{cm}^{-1}$) (c). Fractions were analysed for $\beta_2\text{m}$ by single radial immunodiffusion and shaded areas indicate $\beta_2\text{m}$ -containing fractions that were pooled. Urine from sodium chromate-treated rabbits was concentrated by ultrafiltration and separated by gel chromatography on Sephadex G-100 in 0.02 M TRIS-HCl + 0.15 M NaCl, pH 8.0 (a). The $\beta_2\text{m}$ -containing pool was concentrated and further separated by zone electrophoresis (ZE) in 0.1 M borate buffer, pH 8.9 (b). Eluates corresponding to the larger $\beta_2\text{m}$ peak were pooled, concentrated and submitted to ion-exchange chromatography on DEAE-cellulose (DEAE) with 0.02 M TRIS-HCl, pH 8.0, as starting buffer, followed by a linear salt-gradient elution (0 - 0.15 M NaCl) in the same buffer (c).

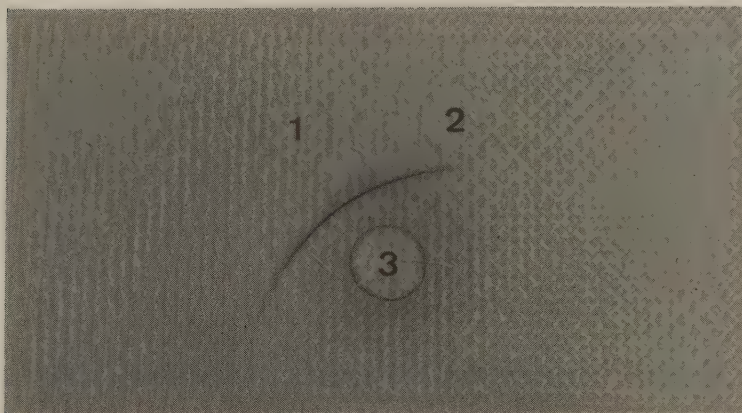


Fig. 3. Ouchterlony immunodiffusion analysis showing a comparison of rabbit (1) and guinea pig (2) β_2m . Well 3 contained goat antiserum against rabbit β_2m .

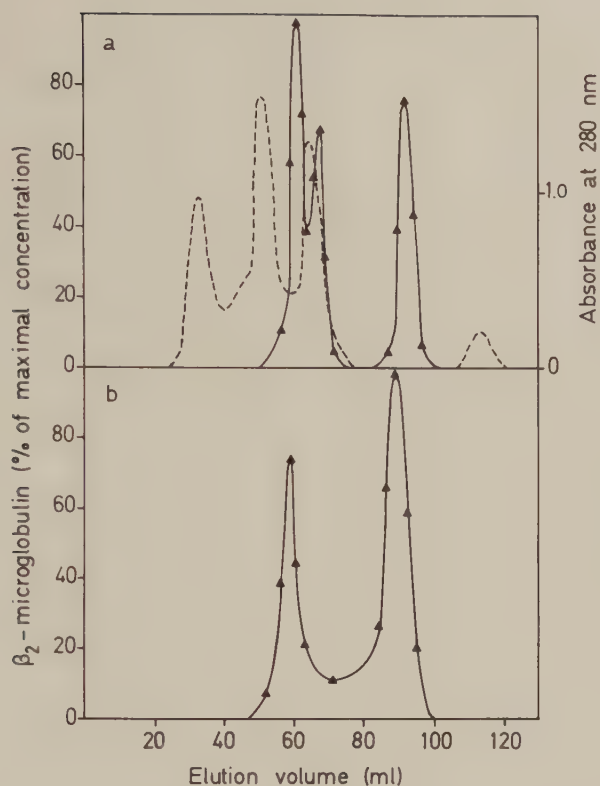


Fig. 4. Gel chromatography on a Sephadex G-200 column (125 x 1 cm) of serum and DOC solubilised splenocyte membrane proteins. Fractions of 0.6 ml were collected at 30-min intervals and analysed for protein (---) and β_{2m} ($\blacktriangle$). Serum samples and splenocytes were taken from the same rabbit. In Fig. 4 a 0.5 ml serum was separated in 0.02 M TRIS-HCl buffer, pH 8.0, containing 0.15 M NaCl. In Fig. 4 b 4.2 mg of splenocyte membrane proteins, solubilised with 0.5 % DOC, were gel filtrated in 0.05 M TRIS-HCl buffer, pH 8.6, containing 0.5 % DOC.

Occurrence of β_2 -microglobulin in mammalian lymphocytes and erythrocytes*

Cell-associated β_2 -microglobulin (β_2 m) has been studied by quantitative techniques in lymphocytes and erythrocytes of guinea pig, rabbit, rat and man. β_2 m was solubilized by sonication followed by treatment with acid, deoxycholate or thiocyanate, and then determined by radioimmunoassay. The average number of β_2 m molecules per lymphocyte, estimated after acidification, varied between 0.89×10^5 and 7.1×10^5 . Rat erythrocytes contained 3.0×10^3 molecules per cell, whereas no β_2 m was detected in red blood cells from the three other species. A relatively large part of β_2 m in the mammalian lymphocytes and in rat erythrocytes was present on the cell surface according to a radioimmunoassay procedure using anti- β_2 m antisera that had been absorbed with the blood cells analyzed.

1 Introduction

Human β_2 -microglobulin (β_2 m) was originally found in free form in various body fluids [1]. Recent work has shown that this small molecule is structurally related to the immunoglobulins [2–4], that it is a cell membrane protein [3, 5], and that it occurs linked to major histocompatibility (HLA-A, B and C) antigens [6–8]. Results from several laboratories suggest that the major serologically defined histocompatibility antigens on mammalian lymphocytes are generally associated with β_2 m (cf. [9]). The major histocompatibility (B) antigens of chicken also appear to be associated with β_2 m because B antigens isolated from leukocytes contain a subunit of β_2 m size [10]. Absolute amounts of β_2 m in lymphoid cells seem to have been determined previously only in such cells from human beings [11, 12]. In mammals, the expression of major histocompatibility antigens on erythrocytes is different in different species. Thus, human erythrocytes apparently are devoid of HLA-A, B and C antigens, with some exceptions [13]. Guinea pig and rabbit erythrocytes have been reported to lack major histocompatibility antigens [14–16], whereas rat erythrocytes carry such antigens [17]. Only human erythrocytes seem to have been analyzed for β_2 m; none was found [11].

In the present work, we have studied the presence of β_2 m on lymphocytes and erythrocytes from guinea pig, rabbit, rat and man. Cells from the four mammals could be analyzed by quantitative techniques, as purified β_2 m from these species and the corresponding antisera were available ([1, 18, 19] and unpublished work by L. Lögdberg, P. O. Östergren and I. Berggård). The results suggest that β_2 m is present in similar numbers in mammalian lymphocytes and that it occurs only on mammalian erythrocytes that express major histocompatibility antigens.

2 Materials and methods

2.1 Separation of lymphocytes and erythrocytes

Mononuclear blood cells were isolated from the different sources (man, rabbit, guinea pig and rat) in the following way. Blood was collected in phosphate-buffered saline, pH 7.4 (PBS), containing 5% dextran 500 (Pharmacia, Uppsala, Sweden). The proportion of blood and dextran solution was 4:1. The dextran solution contained 15 glass beads ($\varnothing$ 2 mm)/ml. Defibrination was carried out by shaking the blood-dextran mixture for 10 min. After sedimentation for 30 min at 37 °C, the supernatant was passed over a cotton wool column [20]. The effluent obtained from the column was subjected to gradient centrifugation [21] on Lymphoprep ($\rho = 1.077$, Nyegaard & Co., Oslo, Norway), at $50 \times g$ for 10 min and at $1000 \times g$ for another 15 min. Cells harvested at the interface region were washed twice with PBS, and smears were stained in Giemsa solution. More than 90% of the cells were identified as lymphocytes.

Erythrocytes used for solubilization of β_2 m or absorption of various anti- β_2 m antisera were prepared as follows. Defibrination and dextran sedimentation were performed as described above. After sedimentation, the bottom fraction was removed and washed once with PBS. Cells were suspended in PBS and passed over a cotton wool column. The effluent was gradient-centrifuged on Lymphoprep at $100 \times g$ for 10 min. The bottom fraction was removed, washed twice and resuspended in PBS followed by another gradient centrifugation. This procedure was repeated once more. Pure erythrocyte suspensions were obtained. Target red cells used for immunofluorescence experiments were collected in heparin and washed twice with PBS.

2.2 β_2 -Microglobulins and antisera

The isolation of human and rabbit β_2 m has been described [1, 18]. Similar procedures were used for preparation of guinea pig [19] and rat β_2 m (Lögdberg, L., Östergren, P. O. and Berggård, I., unpublished). Antisera against rabbit, guinea pig and rat β_2 m were raised in goats, as previously described [9]. Antisera to human β_2 m and normal goat IgG were prepared in rabbits [1]. The anti- β_2 m antisera used gave only a single precipitin line when tested on immunoelectrophoresis against varying amounts of concentrated urinary proteins from sodium chromate-treated rabbits, guinea pigs, rats, or from

[I 2311]

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Abbreviations: β_2 m: β_2 -Microglobulin PBS: Phosphate-buffered saline, pH 7.4 BSA: Bovine serum albumin DC: Deoxycholate

patients with chronic cadmium poisoning. Rabbit anti-goat γ -globulin antisera, conjugated with fluorescein isothiocyanate, were obtained from Behringwerke, Marburg, FRG.

2.3 Preparation of IgG and F(ab')₂ fragments

IgG was prepared from a goat antiserum against rat β_2 m by ion exchange chromatography on DEAE-cellulose (Serva, Heidelberg, FRG) followed by gel filtration on Sephadex G-200 (Pharmacia). F(ab')₂ fragments were obtained from IgG by pepsin digestion. Experimental details have been described previously [9].

2.4 Solubilization of β_2 m from blood cells

The experimental procedure of Evrin and Pertoft [11] was used with minor modifications. The number of lymphocytes submitted to sonication were $7 \times 10^6 - 13 \times 10^6$, and erythrocytes $1 \times 10^9 - 3 \times 10^9$. The cells were suspended in 1.2 ml 0.25 M sucrose containing 0.3% heparin (w/v) (Vitrum, Stockholm, Sweden, 5000 IE/ml). Sonication in this medium was performed with a Branson Sonifier B-12. The suspension was sonicated twice for 10 s. Before and after both sonications the suspension was cooled in an ice water bath. The homogenates were then divided into 4 aliquots of equal volume. To one of the aliquots was added HCl to pH 2.3, and to the remaining three aliquots sodium deoxycholate (DC), NaSCN and Tris-HCl were added giving final concentrations of 1% NaDC + 0.05 M Tris-HCl, pH 8.5, 3 M NaSCN + 0.05 M Tris-HCl, pH 8.5, and 0.05 M Tris-HCl, pH 8.5, respectively. All samples were left at 4 °C for 15 min, after which 3 volumes of 0.05 M Tris-HCl, pH 8.5, + 0.25 M sucrose was added. The aliquot exposed to NaSCN was then dialyzed against 0.05 M Tris-HCl, pH 8.5, + 0.25 M sucrose for 2 h at 4 °C using a 23/32-inch Visking dialysis tubing known to be impermeable to β_2 m [1]. After centrifugation at $105\,000 \times g$ for 1 h 15 min at 4 °C (MSE-65), the supernatants were analyzed for β_2 m.

2.5 Determination of β_2 m

The radioimmunoassay described by Plesner et al. [22] was used. β_2 m homologues were iodinated with ¹²⁵I (Radiochemical Centre, Amersham, GB) according to the chloramine-T method [23]. Iodination was followed by chromatography on Sephadex G-50 (Pharmacia) in order to remove remaining free iodine from the iodinated protein. In most assays, whole antiserum, diluted in PBS containing 0.1% bovine serum albumin (BSA) and 0.02% NaN₃, was used. In some instances, however, IgG and F(ab')₂ fragments to rat β_2 m were also employed. Standard curves, obtained with these reagents, were found to be parallel with the curve acquired with anti-rat β_2 m antiserum, and rat β_2 m determinations of serum samples showed close correlation independently of the antibody source used in the radioimmunoassay.

2.6 Estimation of β_2 m on the cell surface

Lymphocytes and erythrocytes from the different animals were incubated with the corresponding anti- β_2 m antibodies for 3 h at 4 °C. The cell number was estimated in a Bürker chamber. Absorptions were carried out with different amounts of cells.

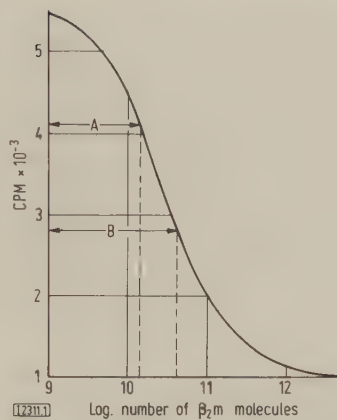


Figure 1. Radioimmunoassay standard curve for human β_2 m. A and B represent the capacity of the same antiserum dilution as used for the standard curve, to bind iodinated β_2 m after absorption of the reagent with human lymphocytes. After absorption of the antiserum with 8×10^4 human lymphocytes (A) it bound 4.1×10^3 cpm which corresponds to 1.4×10^{10} molecules of β_2 m in the standard curve and 1.8×10^5 molecules of β_2 m per human lymphocyte. In (B) 2.5×10^5 lymphocytes were used for absorption giving a value of 1.6×10^5 molecules/cell.

When using lymphocytes, the number varied between 2×10^5 and 1×10^7 , and in the case of erythrocytes, between 1×10^6 and 1×10^9 cells/ml antibody dilution. Absorptions were followed by centrifugation and the supernatant was saved. At the end of the absorption procedure, viability of lymphocytes, as measured by the trypan blue exclusion test, was >90%, while no macroscopical hemolysis was observed in tubes containing erythrocytes.

When estimating the amount of cell-bound β_2 m, the capacity of an absorbed antiserum to bind iodinated β_2 m was compared to a radioimmunoassay standard curve, obtained with the same but unabsorbed antiserum. It was assumed that similar results in the radioimmunoassay would be obtained whether all antibodies reacted with a mixture of unlabeled and labeled antigen, or part of the antibodies were first removed by reaction with unlabeled antigen and the remaining antibodies then reacted with labeled antigen. The procedure is exemplified in Fig. 1 where the standard curve is based on standard solutions of human β_2 m, iodinated human β_2 m, and a rabbit anti-human β_2 m antiserum, diluted 1:50 000 in PBS containing 0.1% BSA and 0.02% NaN₃. In Fig. 1, A and B represent the capacity of this antiserum dilution to bind iodinated human β_2 m following absorption of the reagent with known numbers of lymphocytes. Assuming that each cell-bound β_2 m molecule binds one antibody molecule, the amount of β_2 m on the lymphocytes used for absorption could be calculated from the standard curve. The number of β_2 m molecules/cell was finally determined by dividing this value by the number of cells used for absorption.

2.7 Immunofluorescence procedure

The indirect method was used. One hundred μ l of cells at a concentration of 4×10^6 /ml in PBS containing 0.2% BSA and

0.02% NaN_3 were incubated with an equal volume of IgG solution (concentration varying between 1.7 and 7.4 mg/ml) for 1 h at 20 °C. Cells were washed three times with the buffer mentioned above and then resuspended in 50 μl PBS, pH 7.2. One hundred μl of fluorescein isothiocyanate-conjugated rabbit anti-goat γ -globulin (diluted 1:10 or 1:100 in PBS, pH 7.2) was added to the cell suspension. Incubation for 30 min at 20 °C was followed by another three washes with PBS. Finally, the cells were mounted on slides and observed with the use of a Leitz Ortholux II fluorescence microscope equipped with a Ploem epi-illumination system. Specificity controls included nonimmune goat IgG and inhibition tests with purified $\beta_2\text{m}$.

3 Results

3.1 Quantitation of solubilized $\beta_2\text{m}$ in mammalian lymphocytes and erythrocytes

Solubilization of lymphocytes from guinea pig, rabbit and rat released approximately 10^5 molecules of $\beta_2\text{m}$ per cell, from human lymphocytes somewhat more. In general, treatment of the sonicated cells with HCl or DC released more $\beta_2\text{m}$ than did treatment with thiocyanate. However, DC gave lower yields than thiocyanate from human lymphocytes. Sonication alone resulted in only 15–25% of maximal release of $\beta_2\text{m}$. The results are summarized in Table 1.

When treating erythrocytes with sonication followed by acidification, DC or thiocyanate treatment, apparently no $\beta_2\text{m}$ was released from human, guinea pig or rabbit erythrocytes, while rat erythrocytes yielded 2×10^3 – 3×10^3 molecules/cell, as shown in Table 2. The detection limit, when analyzing for $\beta_2\text{m}$ in red blood cells, was approximately 10 molecules/cell. All values in the tables are calculated on the assumption that $\beta_2\text{m}$ is equally distributed among the cells. Adding HCl, DC or thiocyanate in appropriate concentrations to sera, and purified samples of $\beta_2\text{m}$ of the four species had no significant effect on the antigenicity of $\beta_2\text{m}$, as measured by the radioimmunoassays.

3.2 Quantitation of $\beta_2\text{m}$ on the surface of mammalian lymphocytes and erythrocytes

The number of cell-bound $\beta_2\text{m}$ molecules was estimated using anti- $\beta_2\text{m}$ antibodies absorbed with varying cell amounts. Competition between labeled and unlabeled antigen with the antigen-binding sites on the antibodies is the theoretical basis for the radioimmunoassay. When quantifying $\beta_2\text{m}$ on the cell surface, we compared the standard curve with the decrease in antigen-binding capacity following absorption of the anti- $\beta_2\text{m}$ antibodies. One could object against this direct comparison since there is no competitive situation during the absorption with cell-bound $\beta_2\text{m}$ or when the remaining antibodies react with ^{125}I -labeled $\beta_2\text{m}$. In spite of this, the method was found to be useful and results are summarized in Table 3. The possibility of unspecific binding of antibodies to cells (e.g. Fc receptors) during absorption was studied in the rat system. F(ab')_2 fragments were used for the radioimmunoassay standard curve and for absorptions, and the calculated values were compared to those obtained with intact antibodies. The number of $\beta_2\text{m}$ molecules was found to be 13% lower when F(ab')_2 fragments rather than intact antibodies were absorbed

Table 1. Quantitation of $\beta_2\text{m}$ in lymphocytes of four mammalian species after sonication, followed by acid, DC or thiocyanate treatment

Source of cells ^{a)}	Molecules of $\beta_2\text{m}$ /cell $\times 10^{-5}$ (Mean $\pm$ S.D.)			
	pH 2.3	1% NaDC pH 8.5	3 M NaSCN pH 8.5	pH 8.5
Guinea pig	0.89 (0.32)	1.4 (0.38)	0.59 (0.17)	0.18 (0.08)
Rabbit	1.0 (0.27)	0.93 (0.36)	0.60 (0.18)	0.13 (0.02)
Rat	1.9 (0.14)	1.9 (0.17)	1.5 (0.13)	0.48 (0.09)
Man	7.1 (0.60)	3.5 (0.48)	6.2 (1.0)	1.0 (0.07)

a) Lymphocytes were prepared from ten guinea pigs, three rabbits, eight rats and one human subject.

Table 2. Quantitation of $\beta_2\text{m}$ in erythrocytes of four mammalian species after sonication, followed by acid, DC or thiocyanate treatment

Source of cells ^{a)}	Molecules of $\beta_2\text{m}$ /cell $\times 10^{-3}$ (Mean $\pm$ S.D.)			
	pH 2.3	1% NaDC pH 8.5	3 M NaSCN pH 8.5	pH 8.5
Guinea pig	— ^{b)}	—	—	—
Rabbit	—	—	—	—
Rat	3.0 (0.72)	2.1 (0.30)	2.4 (0.57)	1.2 (0.17)
Man	—	—	—	—

a) Erythrocytes were prepared from ten guinea pigs, three rabbits, eight rats and one human subject.

b) Not detected.

Table 3. Quantitation of cell-bound $\beta_2\text{m}$ on lymphocytes and erythrocytes in four mammalian species. Determinations based on homologous absorptions of anti- $\beta_2\text{m}$ antibodies

Source of cells ^{a)}	Molecules of $\beta_2\text{m}$ /cell (Mean $\pm$ S.D.)	
	Lymphocytes $\times 10^{-5}$	Erythrocytes $\times 10^{-2}$
Guinea pig	0.60 ± 0.33	— ^{b)}
Rabbit	0.66 ± 0.32	—
Rat	0.63 ± 0.18	7.3 ± 2.7
Man	1.3 ± 0.62	—

a) Lymphocytes and erythrocytes were prepared from six guinea pigs, two rabbits, eleven rats and two human subjects.

b) Not detected.

with rat lymphocytes, whereas no significant difference was noticed on rat erythrocytes. The number of $\beta_2\text{m}$ molecules on lymphocytes of the four mammals examined has been adjusted according to these data.

As in the case of $\beta_2\text{m}$ determinations following solubilization, human lymphocytes seem to express the highest absolute amounts of $\beta_2\text{m}$, whereas estimations on lymphocytes from the three rodents indicate small differences between these species. It can also be noticed that values obtained for cell-bound $\beta_2\text{m}$ were consistently lower compared to what was found after solubilization. This could simply be explained by the fact that

different estimation techniques were used. On the other hand, solubilization probably releases cell-bound as well as intracellular β_2 m. Therefore, there is no contradiction in this divergence of obtained results.

Rat erythrocytes were the only red blood cells found to express surface β_2 m. Solubilization or absorption experiments could not answer the question whether only a subpopulation of rat erythrocytes carries β_2 m. Immunofluorescence, however, revealed that 80% of the cells express cell-bound β_2 m. This reaction was specific, since it was completely inhibited by adding purified rat β_2 m to the anti-rat β_2 m IgG solution. Compared to rat lymphocytes, erythrocytes were stained much more weakly.

4 Discussion

β_2 M has been shown to be present on human [3, 5], murine [24], guinea pig and rabbit lymphocytes [9] using qualitative methods. Quantitative determinations of β_2 m have been performed only on human lymphocytes [11, 12]. Human erythrocytes have been reported to lack β_2 m [11], but red blood cells of other species do not seem to have been examined in this respect. In all mammalian species so far investigated, β_2 m has been found to be associated with major histocompatibility antigens (cf. [9]). This also seems to be true for the corresponding antigens (B) in the chicken which have been reported to contain a subunit of β_2 m size [10]. All these studies on the relationship between β_2 m and major histocompatibility antigens have been performed on leukocytes. On erythrocytes, the expression of these antigens seems to differ in different species. Thus, AgB antigens of the rat are present on erythrocytes [17], whereas GPLA antigens of the guinea pig and RLA antigens of the rabbit have not been detected on red blood cells [14-16]. HLA-A, B and C antigens of man, with some exceptions [13], also seem to be absent on erythrocytes. With this background, we found it interesting to investigate the occurrence of β_2 m in erythrocytes, since it was thought that this could provide more information about the expression of this protein in relationship to major histocompatibility antigens.

As already mentioned, β_2 m has previously been quantitated in human blood cells. Evrin and Pertoft [11] found amounts in mononuclear blood cells varying between 4.7×10^5 and 18.1×10^5 molecules/cell depending on the solubilization technique used. Surface-bound β_2 m was calculated to 3.7×10^5 molecules/cell. In close agreement with these data are the determinations performed by Dorval et al. [12] on the surface of human B and T lymphocytes. The amounts of β_2 m on human lymphocytes reported here are somewhat lower but of the same magnitude. In this study, estimations of β_2 m in lymphocytes were extended to three previously not examined mammalian species. Lymphocytes of guinea pig, rabbit and rat were found to have about 10^5 molecules/cell of β_2 m. There was no significant difference between these three rodents, but compared to human lymphocytes, values were lower. The significance of this difference is, however, uncertain since cell sizes, and thereby the densities of β_2 m, were not determined. It should also be mentioned that all quantitative data presented here are based on the assumption that free β_2 m and β_2 m associated with other macromolecules possess identical antigenicities.

Capping experiments performed on lymphocytes of various mammalian species (cf. [9]) indicate that major histocompatibility antigens always carry a β_2 m subunit. When estimating β_2 m and HLA-A, B and C antigens in parallel experiments, Dorval et al. found that the number of β_2 m molecules exceeds the number of HLA molecules [12]. There is, however, no evidence for the presence of free β_2 m on the cell surface. On murine lymphoid cells, β_2 m has been shown to be associated not only with H-2 antigens but also with TL [25, 26] and Qa-2 [27] antigens.

Searching for β_2 m on erythrocytes was thought to be of special interest. Apart from cells of the trophoblastic layer of the placenta [28], erythrocytes are the only normal human cells known to be devoid of β_2 m. They also generally lack HLA-A, B and C antigens. Erythrocytes of the three other mammals studied here had previously not been examined for β_2 m. It was, however, known that AgB antigens are present on rat erythrocytes, whereas GPLA and RLA antigens have not been detected on guinea pig or rabbit red blood cells. In this study, no β_2 m was found on human, guinea pig or rabbit erythrocytes, while rat red blood cells were found to have about 10^3 molecules/cell of β_2 m. Immunofluorescence experiments showed that a majority of rat erythrocytes carries β_2 m. It was noticed that rat lymphocytes were stained much more strongly in these experiments than were rat erythrocytes indicating a lower density on erythrocytes of β_2 m. Although the results on mammalian erythrocytes indicate a parallelism in the expression of β_2 m and major histocompatibility antigens, it should be mentioned that the Daudi cell line, a malignant human hematopoietic cell line cultured *in vitro*, has been reported to synthesize the heavy chain of the HLA antigens [29]. Recently, it has been reported that several other malignant human lymphoma cell lines express β_2 m in low amounts compared to normal lymphocytes, whereas their HLA-A, B and C antigenic determinants were not reduced to the same extent [30]. In spite of this information, our present data suggest a joint expression of β_2 m and major histocompatibility antigens on nonmalignant cells *in vivo*.

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III

Relationships Between β_2 -Microglobulin and Alloantigens Coded for by the Major Histocompatibility Complexes of the Rabbit and the Guinea Pig

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Treatment of rabbit and guinea pig lymphocytes with Fab' fragments of anti- β_2 -microglobulin completely inhibited the cytotoxic effects of alloantisera to RLA or GPLA antigens, respectively. Aggregation of β_2 -microglobulin on the lymphocyte surface by successive incubations with goat anti- β_2 -microglobulin and F(ab')₂ fragments of rabbit anti-goat IgG also made rabbit lymphocytes resistant to lysis by anti-RLA, and guinea pig lymphocytes resistant to lysis by anti-GPLA. The two kinds of pretreatment of guinea pig lymphocytes did not affect the cytotoxicity of antisera directed against guinea pig Ia antigens. These results in conjunction with previous findings in the mouse and in man suggest that β_2 -microglobulin on the lymphocyte surface in mammals is generally associated with major serologically defined histocompatibility antigens but not with I-region-associated antigens.

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The major serologically defined histocompatibility antigens and the I-region-associated (Ia) antigens are (at least in higher vertebrates) determined by genes present in the major histocompatibility complex (35). Previous work on major histocompatibility antigens released from cell surfaces has shown that β_2 -microglobulin (mol.wt., 12,000) is linked to the HLA antigens in man (15, 25, 29), to the H-2 antigens in the mouse (26, 31, 34, 41), and apparently also to the Ag-B antigens in the rat (18). It has been demonstrated that β_2 -microglobulin is associated with HLA and H-2 antigens not only after solubilization of these antigens but also at the cell surface (20, 27, 30, 31, 36). In the mouse, cell surface Ia antigens apparently lack a β_2 -microglobulin subunit (10, 14, 40). This also seems to be

true for Ia-like antigens in man (9, 17). But such a subunit has been found in a murine cell-cell interaction factor that possesses Ia antigenic determinants, and Ia antigens isolated from mouse serum have been reported to contain β_2 -microglobulin (1, 8).

In the present work, we have examined two more animals, the rabbit and the guinea pig, with regard to association of β_2 -microglobulin with alloantigens determined by their major histocompatibility complexes. This study and the previous investigations of human and murine alloantigens (20, 27, 30, 31, 36) suggest that β_2 -microglobulin on intact mammalian lymphocytes is generally associated with the major serologically defined histocompatibility antigens (RLA antigens in the rabbit and GPLA antigens in the guinea pig) but not

with the I-region-associated antigens. According to previous work on guinea pigs, a 12,000-dalton protein occurs bound to solubilized GPLA antigens but not to solubilized Ia antigens (12, 33). Our finding of a GPLA- β_2 -microglobulin association in intact lymphocytes indicates that the 12,000-dalton protein of solubilized GPLA antigens is β_2 -microglobulin.

MATERIALS AND METHODS

Lymphocytes. Lymphocytes from outbred rabbits and guinea pigs were purified from defibrinated blood. After sedimentation for 30 min at 37°C the supernatant was applied to a cotton wool column (19). The effluent obtained from the column was subjected to gradient centrifugation (7) on Lymphoprep, density 1.077 (Nyegaard & Co. A/S, Norway).

β_2 -Microglobulins. The purification of rabbit and guinea pig β_2 -microglobulin from urine of animals treated with sodium chromate has been described (2, 3). Further details of purification and chemical properties will be published later.

Antisera. Antisera against rabbit and guinea pig β_2 -microglobulin were raised in two goats. A mixture of equal volumes of protein solutions (1 mg/ml) and complete Freund's adjuvant (Difco, USA) was inoculated intracutaneously in several sites in the back and intramuscularly in all four legs. Each animal was given 0.4 mg rabbit or guinea pig β_2 -microglobulin. The immunization was repeated 1 month later, and the animals were bled after another 10 days. Incomplete Freund's adjuvant (Difco) was used for the second immunization. The two antisera gave only a single precipitin arc when tested on immunoelectrophoresis against various amounts of concentrated urinary proteins from rabbits or guinea pigs treated with sodium chromate.

Antisera against normal goat IgG (see below) were prepared in two rabbits. The immunization procedure has been described (4).

Alloantisera to RLA antigens 1 and 2 were generous gifts from Dr. H. Matej. These reagents, raised by allogeneic skin graftings, have previously been described by Dr. Matej (22).

Dr. A. L. de Weck kindly supplied us with alloantisera to GPLA antigens B1 and B2 (13, 32) and to guinea pig Ia antigens 1.3 and 3 (13).

Preparation of IgG, F(ab')₂ fragments, and Fab' fragments. Purified IgG from a goat anti-rabbit β_2 -microglobulin antiserum, a goat anti-guinea pig β_2 -microglobulin antiserum, a pooled non-immune goat serum, and a rabbit anti-goat IgG antiserum was obtained by chromatography on DEAE-cellulose (Serva, Germany). A column, 4 × 19 cm, of the ion exchanger was equilibrated with 0.015M Tris-HCl buffer, pH 7.8. Serum dialyzed against the same buffer was applied to the column and eluted at pH 7.8 with a 4000-ml linear gradient of NaCl from 0 to 0.2M. Protein eluted up to 0.06M NaCl was concentrated by ultrafiltration and analyzed by agarose gel electrophoresis (16). Only one band in the gamma-globulin region was observed. F(ab')₂ fragments from the IgG preparations were obtained by pepsin digestion (28) followed by chromatography on Sephadex G-200 (Pharmacia, Sweden). Fab' fragments were prepared by reduction and alkylation of the F(ab')₂ fragments, again followed by chromatography on Sephadex G-200 (39).

Lymphocytotoxicity testing. A two-step microcytotoxicity test was used (37). One microliter of antiserum and 1 μ l of cell suspension (2000 cells) were incubated under liquid paraffin (ACO, Sweden) on microculture plates (Møller-Coates, Norway) for 30 min at 20°C. Five microliters of rabbit and guinea pig serum in a 2:1 proportion were added as complement source. Incubation with complement proceeded for 1 h at 20°C. The plates were read with an inverted microscope after addition of 2 μ l of 1% trypan blue in saline. The reactions were scored as follows: 0%–10% dead cells: – ; 10%–20%: + ; 20%–50%: ++ ; 50%–75%: +++ ; and 75%–100%: ++++.

Inhibition tests were performed by adding β_2 -microglobulin to the anti- β_2 -microglobulin antisera and by pretreating lymphocytes with Fab' fragments. In the latter case, lymphocytes were pretreated with Fab' fragments by two different techniques. First, 0.5 ml of lymphocytes in phosphate-buffered saline (PBS), pH

Table I. Effects of Fab' fragments of anti-rabbit β_2 -microglobulin on the lymphocytotoxicity induced by anti-RLA and anti- β_2 -microglobulin antisera

Sera	Rabbit 1 lymphocytes*		Rabbit 2 lymphocytes*	
	Untreated	Fab'-treated	Untreated	Fab'-treated
Anti-RLA 1	++	-	-	-
Anti-RLA 2	+++	-	++	-
Anti-rabbit β_2 -microglobulin	++++	-	++++	-
Non-immune rabbit or goat sera	-	-	-	-

* - = 0%-10% lysis, + = 10%-20% lysis, ++ = 20%-50% lysis, +++ = 50%-75% lysis, ++++ = 75%-100% lysis.

7.4, at a concentration of $1 \cdot 10^6$ cells/ml were incubated with an equal volume of Fab' fragments at a concentration of 3.0 mg/ml for 1 h at 4°C. The lymphocytes were washed four times in ice-cold PBS, and the cell concentration was adjusted to $2 \cdot 10^6$ cells/ml. Second, 1 μ l of Fab' fragments at a concentration of 3.0 mg/ml and 1 μ l of lymphocyte suspension (2000 cells) were incubated under liquid paraffin on microculture plates for 1 h at 20°C.

These two methods gave the same results when the pretreated lymphocytes were submitted to microcytotoxicity testing as described above. The second technique has the advantage of demanding less reagent, time, and labor.

Aggregation of cell surface β_2 -microglobulin. The technique used, often called the lysostrip method, has been described previously (5). Lymphocytes were first incubated with goat anti- β_2 -microglobulin antiserum (heat-inactivated and dialyzed against PBS, pH 7.4) for 2 h at room temperature and washed three times with ice-cold PBS. 0.3 ml of cells ($4 \cdot 10^6$ /ml) and 0.3 ml of rabbit anti-goat IgG F(ab') fragments (15 mg/ml) were allowed to react for 30 min at 0°C. Cells were washed three times with ice-cold PBS, and the concentration was adjusted to $2 \cdot 10^6$ /ml in RPMI-1640 (Flow Laboratories, U.K.). After another 30 min of incubation at 37°C the lymphocytes were quickly cooled to 0°C and 1M NaN₃ in PBS was added to the cells, giving the suspension a NaN₃ concentration of 10mM. Finally, the lymphocytes were subjected to cytotoxicity testing as previously described.

RESULTS

The relationship between β_2 -microglobulin and RLA antigens

Lymphocytes from eight rabbits were tested against a goat anti-rabbit β_2 -microglobulin antiserum. The antiserum was clearly cytotoxic to lymphocytes from all rabbits examined. The specificity of the reaction was studied by adding purified rabbit β_2 -microglobulin to the goat antiserum. This preincubation completely abolished the cytotoxicity of the reagent. Pretreatment of the lymphocytes with Fab' fragments against rabbit β_2 -microglobulin had the same effect (Table I), whereas Fab' fragments prepared from non-immune goat IgG did not influence the cytotoxic property of the antiserum.

By using anti-RLA antisera it was possible to classify the eight rabbits into RLA 1,2, RLA 2, and RLA 0 animals. Lymphocytes from rabbits of different specificities were pretreated with goat Fab' fragments against rabbit β_2 -microglobulin. As shown in Table I, this made the cells resistant to lysis by anti-RLA. Fab' fragments from non-immune goat IgG were used as a control. In this case no inhibition of anti-RLA lymphocytotoxicity was observed.

Further evidence of an association between β_2 -microglobulin and RLA was obtained by using the lysostrip technique. Aggregation of β_2 -microglobulin on the lymphocyte surface by successive incubations with goat anti-rabbit β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG effectively protected the cells against lysis by anti-RLA (Table II).

Table II. Inhibition of anti-RLA- and anti- β_2 -microglobulin-induced cytotoxicity on rabbit lymphocytes by redistribution of β_2 -microglobulin on the cell surface

Sera	Rabbit 3 lymphocytes*		Rabbit 4 lymphocytes*	
	Untreated	Pretreated†	Untreated	Pretreated†
Anti-RLA 1	++	—	—	—
Anti-RLA 2	++	—	+++	—
Anti-rabbit β_2 -microglobulin	++++	—	++++	—
Non-immune rabbit or goat sera	—	—	—	—

* See Table I for explanation of reaction scores.

† β_2 -microglobulin on the cells was redistributed by successive incubations with goat anti-rabbit β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG.

Association of β_2 -microglobulin with GPLA and guinea pig Ia antigens

The experimental procedures described above were used in experiments with lymphocytes from 32 guinea pigs. Specificity tests included absorption of the goat anti-guinea pig β_2 -microglobulin antiserum with purified guinea pig β_2 -microglobulin and inhibition on the lymphocyte surface with Fab' fragments of anti- β_2 -microglobulin IgG. In both cases, cytotoxicity was brought down to background levels. On the other hand, non-immune goat Fab' fragments did not affect the reactivity of the anti- β_2 -microglobulin antiserum, the anti-GPLA antisera, or the anti-Ia antisera.

Guinea pig lymphocytes pretreated with Fab' fragments against β_2 -microglobulin or manip-

ulated according to the lysostrip technique became resistant to anti-GPLA cytotoxicity, whereas the capability of anti-Ia to cause lympholysis remained unaffected. The results, summarized in Tables III and IV, indicate an association between GPLA and β_2 -microglobulin on the cell surface, whereas guinea pig Ia antigens do not seem to be bound to β_2 -microglobulin.

DISCUSSION

Previous work has shown that β_2 -microglobulin is part of the human HLA antigens (15, 20, 25, 27, 29, 30, 36), the murine H-2 antigens (26, 31, 34, 41), and probably also the rat Ag-B antigens (18), whereas murine mem-

Table III. Effects of Fab' fragments of anti-guinea pig β_2 -microglobulin on the lymphocytotoxicity induced by anti-GPLA, anti-Ia, and anti- β_2 -microglobulin antisera

Sera	Lymphocytes* from					
	Guinea pig 1		Guinea pig 2		Guinea pig 3	
	Untreated	Fab'-treated	Untreated	Fab'-treated	Untreated	Fab'-treated
Anti-GPLA B.1	+++	—	++	—	—	—
Anti-GPLA B.2	—	—	—	—	++	—
Anti-Ia 1.3	++	++	++	++	—	—
Anti-Ia 3	++	++	—	—	—	—
Anti-guinea pig β_2 -microglobulin	++++	—	++++	—	++++	—
Non-immune guinea pig or goat sera	—	—	—	—	—	—

* See Table I for explanation of reaction scores.

Table IV. Inhibition of anti-GPLA-, anti-Ia-, and anti- β_2 -microglobulin-induced cytotoxicity on guinea pig lymphocytes by redistribution of β_2 -microglobulin on the cell surface

Sera	Lymphocytes* from					
	Guinea pig 4		Guinea pig 5		Guinea pig 6	
	Untreated	Pre-treated†	Untreated	Pre-treated†	Untreated	Pre-treated†
Anti-GPLA B.1	+++	-	-	-	-	-
Anti-GPLA B.2	-	-	+++	-	+++	-
Anti-Ia 1.3	-	-	+	+	++	++
Anti-Ia 3	-	-	-	-	++	++
Anti-guinea pig β_2 -microglobulin	+++	-	+++	-	+++	-
Non-immune guinea pig or goat sera	-	-	-	-	-	-

* See Table I for explanation of reaction scores.

† β_2 -microglobulin on the cells was redistributed by successive incubations with goat anti-guinea pig β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG.

brane-bound Ia antigens and human Ia-like antigens seem to lack this subunit (9, 10, 14, 17, 40). We have investigated the possible association between β_2 -microglobulin and the major serologically defined histocompatibility antigens in two additional mammalian species, the rabbit and the guinea pig. The possible relationship between β_2 -microglobulin and guinea pig Ia antigens has also been examined.

The major histocompatibility system in the rabbit has been studied by several investigators (6, 11, 21, 22, 38). There have been considerable difficulties in finding an effective genetic approach to this histocompatibility system, mainly because of the lack of homozygous rabbit strains. Despite this, alloantisera have been raised in rabbits which detect alloantigens that most likely represent the rabbit counterpart of the murine H-2 or the human HLA system. The antisera used in our experiments detect the RLA 1 and RLA 2 antigens, which are well-defined antigens with relatively broad specificities. They are apparently determined by genes present at two different but closely linked loci (H. Matej, personal communication). The tissue distribution of RLA antigens (21, 23) and the fact that compatibility of these antigens prolongs the survival of skin allografts in related rabbits (24) are in analogy with the HLA and H-2 antigens. So far, no chemical

data are available concerning the structure of the RLA antigens. The results of this study indicate that the RLA antigens have a β_2 -microglobulin subunit and a basic structure similar to the structure of the H-2 and the HLA antigens.

The GPLA antigens were originally discovered by using alloantisera produced by cross-immunization of outbred guinea pigs (32). The structural characteristics of these antigens have been the subject of recently published studies (12, 33). Solubilized GPLA molecules were found to be composed of two subunits. The antigenic determinants were located to a 40,000-dalton glycoprotein. Non-covalently associated with this larger part of the molecule was a 12,000-dalton protein component. These data are in close agreement with studies concerning the subunit structure of solubilized H-2 and HLA antigens, where the 12,000-dalton component has been proved to be β_2 -microglobulin. In this paper we present strong evidence of a linkage between GPLA antigens and β_2 -microglobulin on intact cells. Our findings and the observation that one of our anti- β_2 -microglobulin sera precipitates both a 11,000- and a 40,000-dalton membrane protein (E. M. Shevach, personal communication) seem to establish that GPLA antigens are associated with β_2 -microglobulin.

Our present knowledge on the genetics and the structural characteristics of Ia antigens in the guinea pig has developed rapidly during the last 2 years (12, 13, 33). It has been reported that guinea pig Ia antigens, solubilized from cell surfaces, do not contain any subunit of β_2 -microglobulin size (12, 33). In agreement with this observation, we did not find any evidence of an association between Ia antigens and β_2 -microglobulin on intact cells, since blocking or aggregation of cell surface β_2 -microglobulin did not affect the activity of anti-Ia antisera. Previous studies have shown that murine cell surface Ia antigens apparently also lack a β_2 -microglobulin subunit (10, 14, 40). But Ia antigens isolated from serum (8) and culture supernatants (1) have been reported to contain β_2 -microglobulin. There is no obvious explanation of this discrepancy in the structure of cell-bound and free Ia antigens. One could, however, speculate about the possibility that a subpopulation of cell surface Ia antigens, carrying β_2 -microglobulin, is not detectable by present methods. Ia antigens associated with β_2 -microglobulin could also, hypothetically, represent molecules derived from secreting cells. Finally, secreted and/or shed Ia antigens might change their conformations in a way that favors an extracellular association with β_2 -microglobulin.

Previously published data on the structure of HLA and H-2 antigens, in combination with the results presented in this study and in the work of Schwartz et al. (33), make it reasonable to suggest that, in mammals, the major serologically defined histocompatibility antigens are generally associated with β_2 -microglobulin, whereas at least some cell-bound Ia antigens do not have a β_2 -microglobulin subunit.

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HETEROLOGOUS INTERACTION BETWEEN β_2 -MICROGLOBULIN AND Ag-B ANTIGENS¹

Classical major histocompatibility antigens (HLA-A,B,C in man, H-2-D,K (L) in mouse, Ag-B in rat, etc.)—H antigens—seem to be critically involved as target molecules in the destruction of foreign or autologous cells by cytolytic T lymphocytes (1). The H antigens are integral membrane glycoproteins situated on most cell types. They are composed of two noncovalently associated polypeptide chains (2). The heavy chain (mol wt ~ 44,000) carries the alloantigenic determinants, the carbohydrate moiety, and is responsible for the membrane penetration. The light chain (mol wt ~ 12,000) is a water-soluble protein (β_2 -microglobulin (β_2 M)) first isolated by Berggård (3) from pathological urine. Early suggestions that H antigens and immunoglobulins might share a common evolutionary origin (4, 5) have been substantiated by amino acid sequence analyses

TABLE 1. Binding of radiolabeled homologous and heterologous β_2 M by purified Ag-B antigens^a

Step 1: incubation	Step 2: Precipitating serum		
	Normal BN	WF anti-BN	BN anti-WF
Experiment A			
¹²⁵ I-Rat β_2 M + Ag-B + 0	739	778	2,406
¹²⁵ I-Rat β_2 M + Ag-B + Rat β_2 M ^b	808	864	745
¹²⁵ I-Rat β_2 M + Ag-B + Hum β_2 M	772	750	792
¹²⁵ I-Rat β_2 M + Ag-B + Rab β_2 M	795	774	744
¹²⁵ I-Rat β_2 M + Ag-B + GP β_2 M	760	855	776
¹²⁵ I-Rat β_2 M + Ag-B + Hum α_1 M	824	766	2,423
¹²⁵ I-Rat β_2 M + 0 + 0	766	824	812
Experiment B			
¹²⁵ I-Hum β_2 M + Ag-B + 0	2,238	2,465	16,022
¹²⁵ I-Hum β_2 M + Ag-B + Rat β_2 M	2,644	2,652	2,759
¹²⁵ I-Hum β_2 M + Ag-B + Hum β_2 M	2,877	2,680	2,741
¹²⁵ I-Hum β_2 M + Ag-B + Rab β_2 M	2,749	2,892	2,794
¹²⁵ I-Hum β_2 M + Ag-B + GP β_2 M	2,620	2,566	2,848
¹²⁵ I-Hum β_2 M + Ag-B + Hum α_1 M	2,593	2,660	14,870
¹²⁵ I-Hum β_2 M + 0 + 0	3,026	3,030	3,108
Experiment C			
¹²⁵ I-Rab β_2 M + Ag-B + 0	1,878	2,186	6,158
¹²⁵ I-Rab β_2 M + Ag-B + Rat β_2 M	2,158	2,142	1,637
¹²⁵ I-Rab β_2 M + Ag-B + Hum β_2 M	2,044	2,324	2,281
¹²⁵ I-Rab β_2 M + Ag-B + Rab β_2 M	1,856	2,058	2,038
¹²⁵ I-Rab β_2 M + Ag-B + GP β_2 M	1,854	1,968	1,798
¹²⁵ I-Rab β_2 M + Ag-B + Hum α_1 M	2,200	2,254	5,788
¹²⁵ I-Rab β_2 M + 0 + 0	2,026	2,238	2,278
Experiment D			
¹²⁵ I-GP β_2 M + Ag-B + 0	2,925	2,834	9,976
¹²⁵ I-GP β_2 M + Ag-B + Rat β_2 M	3,143	3,036	3,179
¹²⁵ I-GP β_2 M + Ag-B + Hum β_2 M	3,120	3,107	2,764
¹²⁵ I-GP β_2 M + Ag-B + Rab β_2 M	3,039	2,850	2,909
¹²⁵ I-GP β_2 M + Ag-B + GP β_2 M	2,822	2,704	2,867
¹²⁵ I-GP β_2 M + Ag-B + Hum α_1 M	3,216	2,922	9,547
¹²⁵ I-GP β_2 M + 0 + 0	3,002	2,846	2,950
Experiment E			
¹²⁵ I-Hum α_1 M + Ag-B + 0	483	462	490
¹²⁵ I-Hum α_1 M + 0 + 0	540	529	508

^a Results are mean cpm values for two aliquots. An estimation of the technical error, based on variation within duplicates from several experiments, gave a SD value of 4.9% (269 df). Total cpm for the aliquots were 22,000 in experiment A, 68,000 in experiment B, 69,000 in experiment C, 61,000 in experiment D, and 25,000 in experiment E.

^b In some incubations, unlabeled proteins, β_2 Ms or α_1 M at 10 μ g/ml, were included. Hum, human; Rab, rabbit; GP, guinea pig.

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TABLE 2. Inhibition of interaction between human β_2 M and Ag-B antigens by heterologous sera^a

Step 1: incubation	Step 2: precipitating serum		
	Normal BN	BN anti-WF	Specific precipitation (Δ cpm) (%)
Experiment A			
¹²⁵ I-Hum β_2 M + Ag-B + 0	2,868 ^b	15,562	12,694 100
¹²⁵ I-Hum β_2 M + Ag-B + cow serum (1:10)	4,386	4,476	90 0.7
¹²⁵ I-Hum β_2 M + Ag-B + rhesus monkey serum (1:10)	3,599	3,794	195 1.5
¹²⁵ I-Hum β_2 M + Ag-B + mouse serum (1:10)	4,935	5,144	209 1.6
Experiment B			
¹²⁵ I-Hum β_2 M + Ag-B + 0	2,496	14,850	12,354 100
¹²⁵ I-Hum β_2 M + Ag-B + spiny dogfish serum (1:10)	3,731	15,848	12,117 98
¹²⁵ I-Hum β_2 M + Ag-B + G-200 III $\times$ 8 (1:10)	3,414	12,274	8,860 72

^a In experiment A, sera from cow, rhesus monkey, or mouse were included in the incubation mixtures. In experiment B, incubation mixtures contained serum or a concentrated pool (G-200 III) of Sephadex G-200 chromatographed serum from spiny dogfish (*S. acanthius*). The pool corresponded to K_{av} values 0.10 to 0.25 and was concentrated to a volume 1/8 of the separated serum before use. The other pools of the separated fish serum did not markedly block the specific precipitation (<10% inhibition).

^b Results are mean cpm values for two aliquots. Technical error $\approx$ 4.9% (see Table 1).

of β_2 M (6, 7) and, more recently, of the H antigen heavy chain (8, 9). Accordingly, β_2 M and the H antigen heavy chain have been well conserved through evolution, as shown by sequence studies and immunological cross-reactions in various mammalian species and chicken (10-12). The present work demonstrates this evolutionary conservation also by heterologous interactions between β_2 M and the H antigen heavy chain. The results suggest that these types of interactions could be a tool in pursuing H antigen analogues in lower species.

Binding of radiolabeled β_2 M to purified Ag-B antigens was carried out as follows: Step 1: Purified papain-solubilized splenocyte Ag-B antigens (~5 ng/ml; estimated from β_2 M content) from inbred Wistar-Furth (WF) rats (L. Björck, B. Axelsson, and L. Löfdberg, unpublished data) and radiolabeled β_2 M (2 to 10 ng/ml) from rat (12), man (13), rabbit (14), or guinea pig (15) were mixed and incubated at 37 C for 24 hr. This procedure has been shown to allow binding of radiolabeled rat β_2 M to high molecular weight complexes in rat serum (12), and also binding of β_2 M to high molecular weight complexes in heterologous sera (L. Löfdberg, R. Cigén, and L. Björck, unpublished observations). Step 2: Antiserum specific for WF alloantigens, raised in inbred Brown Norway (BN) rats (BN anti-WF) or control sera (WF anti-BN or normal BN), were added (25 μ l) to aliquots (200 μ l) of the reaction mixtures. Incubation was proceeded at room temperature for at least 5 hr. Step 3: Immune complexes were separated from unbound β_2 M by polyethylene-glycol exclusion (16) and the radioactivity in the precipitate was determined.

Binding of radiolabeled rat β_2 M to Ag-B antigens was demonstrated as specific precipitation of radioactivity by alloantisera directed against the relevant Ag-B antigens, which was lost if the Ag-B antigens were excluded from step 1 (Table 1, A). The specificity of the binding was confirmed as it could be completely blocked by unlabeled rat β_2 M but not by the pre-

sumably irrelevant protein human α_1 -microglobulin (α_1 M). This suggests that radiolabeled rat β_2 M is exchanged with the light chain of the Ag-B antigens. Recent studies in the human system (17) support this conclusion. It was now very interesting to find that this binding could also be completely blocked by β_2 M from three other species (Table 1, A). These findings are interpreted as an evolutionary conservatism in the β_2 M-binding site on the H antigen heavy chain and the corresponding determinants on β_2 M. Moreover, the Ag-B antigens could also specifically bind radiolabeled β_2 M from man, rabbit, or guinea pig (Table 1, B to D). These heterologous bindings of β_2 M could always be completely blocked by unlabeled β_2 M from the four species, but never by human α_1 M. Predictably, Ag-B antigens could not bind radiolabeled human α_1 M (Table 1, E). These results corroborate and extend recent observations by Hester et al. (18), which suggested that H 2 antigens on mouse lymphocytes could act as a receptor for human β_2 M.

β_2 M seems to influence the conformation of H antigens (19). Thus, the exchange of homologous for heterologous β_2 M in the H antigen molecule might lead to altered conformation; however, no effect on allospecificity is apparent, as alloantisera were used in this study.

The heterologous binding of β_2 M by H antigens could be expected to be useful for detection and purification of analogous molecules in species where these reagents are not available. Sera from cow, rhesus monkey, and mouse abrogated the binding of radiolabeled human β_2 M to Ag-B antigens (Table 2, A), supporting this assumption. These sera did not interfere with the binding of radiolabeled Ag-B antigens by BN anti-WF alloantibodies (not shown). Thus, their inhibitory activity is specifically exerted on the heterologous Ag-B β_2 M interaction.

Evolutionary studies on β_2 M and H antigens should have strong implications on our understanding of the evolution of the immune system. However, unambiguous biochemical demonstration of these molecules has, as far as we know, only been achieved in mammals and birds. Therefore, it is very promising to find that a concentrated pool of Sephadex G-200-separated serum from spiny dogfish (*Squalus acanthius*) can partially inhibit the Ag-B-binding of radiolabeled human β_2 M (Table 2, B). Such inhibition is also executed by a similar concentrated pool of gel chromatographed serum from an aglomerular fish (*Lophius americanus*) (specific precipitation, 65%). Gordon and Kindt (20) previously found heterologous β_2 M radioimmunoassay inhibitory activity in sera from lower vertebrates. In our hands, however, such activity is restricted to the void fraction after Sephadex G-200 chromatography. This is in contrast to

the molecules responsible for the inhibition of the Ag-B- 125 I-labeled human β_2 M interaction, which, both in *S. acanthius* and *L. americanus*, are included in the separation range of Sephadex G-200.

In summary, we have demonstrated a heterologous interaction between H antigens and β_2 M that promises to be a valuable tool for biochemical studies of H antigens in lower species.

Note added in proof. Since the submission of this manuscript, we have found that purified papain-solubilized Ag-B antigens from inbred BN rats show the same kind of reactivity with heterologous β_2 M as Ag-B antigens from WF rats.

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PROLONGED SURVIVAL OF HEART ALLOGRAFTS IN RATS TREATED WITH ANTISERA TO β_2 -MICROGLOBULIN¹

β_2 -microglobulin has been the subject of numerous recent studies because of its homology with immunoglobulin domains, its association with histocompatibility antigens, and its possible association with lymphocyte receptor structures (7, 13). Antibodies against β_2 -microglobulin have been shown to inhibit several lymphocyte proliferative responses, such as the mixed lymphocyte culture reaction (1, 10, 11, 17), the stimulation with phytohemagglutinin (1, 16), and the stimulation with the soluble antigen-purified protein derivative of tuberculin (1, 11, 17). These reactions are known to be carried out primarily by thymus-derived (T) lymphocytes. According to one investigation (2), anti- β_2 -microglobulin also seems to inhibit the T lymphocyte effector function determined by the cell-mediated lympholysis assay.

Because antisera against β_2 -microglobulin repress various T lymphocyte reactions in vitro, it seemed to be of interest to examine whether such antisera also can delay the allograft rejection process which is largely effected by T lymphocytes.

We have studied the effect of treatment with anti- β_2 -microglobulin sera on the survival of heart allografts transplanted between two isogenous inbred rat strains. Hearts from male Wistar-Furth (WF) rats (Ag-B2) were grafted heterotopically into the peritoneal cavity of male 250- to 300-g animals of the Brown Norway (BN) strain (Ag-B3). The surgical technique of Ono and Lindsay was used (14). Graft survival was determined by daily palpations and, if necessary, by laparotomy. Isografts survived for several months (upper limit not determined). Two technical failures (nonbeating heart 24 hr after surgery) were recorded.

A goat anti-rabbit β_2 -microglobulin serum (4) was used in the first series of experiments. This antiserum was found to be cytotoxic not only to rabbit lymphocytes, but also to rat lymphocytes (L. Björck, B. Löw, and I. Berggård, unpublished data) and to precipitate purified rat β_2 -microglobulin on immunodiffusion analyses (L. Lögdberg, P. O. Östergren, and I. Berggård, unpublished data). A second series of experiments was performed with a rabbit anti-

serum (5) against rat β_2 -microglobulin. In a two-step lymphocytotoxicity test (18), the cytotoxic titres of the two antisera (dilutions inducing 50% lysis of BN lymphocytes) were 1:8 (goat anti-rabbit β_2 -microglobulin serum) and 1:16 (rabbit anti-rat β_2 -microglobulin serum). Both antisera showed only one precipitin arc when tested on immunoelectrophoresis against varying amounts of concentrated urinary rabbit or rat proteins.

Control groups of rats received pooled nonimmune goat serum, pooled nonimmune rabbit serum, or rabbit anti-human albumin serum. All three control sera were noncytotoxic to BN lymphocytes. The rabbit anti- β_2 -microglobulin and antialbumin sera were obtained by the same immunization procedure (5).

The anti-BN erythrocyte agglutination titres were 1:4, 1:8, and 1:16 in all three rabbit sera, the goat anti-rabbit β_2 -microglobulin serum, and the pooled nonimmune goat serum, respectively.

All sera were heat-inactivated at 56 C for 30 min, dialyzed against phosphate-buffered saline, pH 7.4, and passed through Swinnex-25 filters (Millipore). One-ml portions of sera were given i.v. as indicated in Table 1.

The transplant survival times are summarized in Table 1. Twenty-five rats were grafted without serum treatment. The functional graft survival time was 6.84 ± 0.14 days (mean $\pm$ SD). The survival of the allografts in five animals that received goat anti-rabbit β_2 -microglobulin and in five animals that received rabbit anti-rat β_2 -microglobulin was in both instances 8.60 ± 0.80 days. The difference in graft survival between groups that were given anti- β_2 -microglobulin sera and nonimmune sera was statistically analysed by the Mann-Whitney U test. The survival time after anti- β_2 -microglobulin serum administration was significantly prolonged ($P < 0.01$).

The prolongation of allograft survival is comparatively small (8). One possible explanation for this could be absorption of antibodies by β_2 -microglobulin present on the recipients non-lymphoid tissues, thereby leaving only a small amount of anti- β_2 -microglobulin to procure allograft survival.

In several studies permanent graft survival has been induced by enhancing alloantisera or

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TABLE 1. Survival of heart transplants after treatment with anti- β_2 -microglobulin sera, rabbit anti-human albumin serum, and nonimmune sera^a

Goat anti-rabbit β_2 -microglobulin serum	Graft survival, days for each serum-treated rat			Rabbit anti-human albumin serum
	Pooled nonimmune goat serum	Rabbit anti-rat β_2 -microglobulin serum	Pooled nonimmune rabbit serum	
8	7	8	7	7 ^b
9	6	9	7	7 ^b
8	7	8	7	
8	7	10 ^c	7 ^b	
10 ^c	7 ^b	8 ^c	7 ^b	
Mean $\pm$ SD 8.6 $\pm$ 0.8	6.8 $\pm$ 0.2	8.6 $\pm$ 0.8	7 $\pm$ 0	7 $\pm$ 0

^a Except where indicated the recipient was given 1 ml of serum on the day before operation, 1 ml at the time of transplantation (day 0), and 1 ml on days 1 to 6 (total dose = 8 ml).

^b Treatment was proceeded until rejection (total dose = 9 ml).

^c Treatment was proceeded for another 2 days (total dose = 10 ml).

antilymphocyte sera in certain rat strain combinations. In these experiments, however, semi-allogeneic (F₁) donors have usually been used. The survival times have been considerably reduced when the gene dose differences between donors and recipients have been increased (8).

The WF-BN histocompatibility barrier is strong (3). This was also observed by grafting hearts from WF rats into six BN animals treated with a rabbit anti-BN lymphocyte serum (9) in a similar protocol. The prolongation of transplant survival time was 2 to 4 days (P. Konrad and B. Husberg, unpublished data). This is only slightly more than the prolongation caused by anti- β_2 -microglobulin. It should be possible to achieve longer allograft survival times with anti- β_2 -microglobulin by the use of weaker histocompatibility barriers. However, the purpose of this study was more to establish that anti- β_2 -microglobulin has graft protective properties than to determine the strength of the immunosuppressive effect.

As described above, all sera used in this study had low titres of agglutinins against BN rat erythrocytes. These agglutinins do not seem to influence the survival of the allografts, since the time of rejection was the same in untreated rats and in animals treated with nonimmune or antihuman albumin sera.

Antisera against human β_2 -microglobulin have been reported to be mitogenic to bone marrow-derived (B) lymphocytes in vitro (12, 15, 16). Such effects can hardly explain the prolonged allograft survival observed in this study.

T lymphocyte-mediated immune reactions are regarded as the most important in the rejection of first-set transplants. Antibodies to β_2 -

microglobulin could interfere with the rejection process by reacting with responding T lymphocytes, with the allograft or with both.

It seems likely that the prolonged allograft survival observed in this study is at least partly attributable to interference with T lymphocyte functions. As mentioned above, it has been shown that antibodies against β_2 -microglobulin inhibit the mixed lymphocyte culture (1, 10, 11, 17) and cell-mediated lympholysis (2) reactions and also the antigenic stimulation of lymphocytes with purified protein derivative (1, 11, 17). Studies from two laboratories indicate that anti- β_2 -microglobulin inhibits the responding cells in the mixed lymphocyte culture reaction (10, 11). The combined evidence from all of these experiments suggests that β_2 -microglobulin is part of, or associated with, receptor structures on T lymphocytes. Anti- β_2 -microglobulin could act by blocking or modulation of such receptor structures and by complement-dependent cytotoxicity. The possible contribution of cytotoxicity can be explored by treatment of allograft recipients with F(ab')₂ fragments of anti- β_2 -microglobulin.

It is an established fact that β_2 -microglobulin is part of the major serologically defined transplantation antigens (7, 13). Thus, anti- β_2 -microglobulin might also act as enhancing antibodies by binding to such antigens in the transplant. The nature of enhancing antibodies in the rat model is, however, not clear. According to a recently published report, the predominant part of such antibodies do not have specificity to the Ag-B antigens, but rather to antigenic structures that may represent Ia specificities (6). β_2 -microglobulin apparently is not associated with Ia antigens (19).

In summary, antibodies against β_2 -microglobulin have been shown to cause a significant but moderate prolongation of the survival of rat heart allografts. It is suggested that the prolongation is at least partly attributable to interference with T lymphocyte functions.

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Binding of Aggregated Human β_2 -Microglobulin to Surface Protein Structure in Group A, C, and G Streptococci

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A novel mammalian-microbial "short circuit" has been demonstrated between aggregated human β_2 -microglobulin and group A, C, and G streptococci. Bacteria belonging to nine gram-positive and three gram-negative species were tested for binding of radiolabeled β_2 -microglobulin. All 10 individual strains of group A streptococci showed a high degree of reactivity with aggregated human β_2 -microglobulin. Among 27 group C and 28 group G streptococci, 9 and 6 strains, respectively, were highly reactive, whereas the remaining strains showed a lower, but definite level of β_2 -microglobulin binding. Of 11 group B streptococci, 4 were slightly positive. All strains among the other eight species were completely negative. Simultaneous testing of A, C, and G streptococci for immunoglobulin binding showed a lack of correlation between type II and III Fc reactivity and β_2 -microglobulin binding. There was no inhibition of uptake of aggregated β_2 -microglobulin to reactive strains when excess amounts of human immunoglobulin were added. The β_2 -microglobulin-binding surface structure was found to be markedly sensitive to trypsin digestion. The relative trypsin resistance of the immunoglobulin-binding protein in the digestion experiments further demonstrated the dissociation between these two reactivities.

β_2 -microglobulin is a small protein originally isolated from human urine (4). It is similar to immunoglobulin domains with respect to size and amino acid sequence (16, 18), and it occurs linked to major histocompatibility antigens of various mammalian species (cf. reference 5). These observations and the fact that the larger subunits of such antigens (HLA and H2) seem to be arranged in domains (17, 19) suggest a structural relationship between immunoglobulins and major histocompatibility antigens. Similarities have also been shown in the expression of functional characteristics. Aggregated human β_2 -microglobulin was capable of fixing complement, and, when coated onto sheep erythrocytes, human β_2 -microglobulin promoted binding to guinea pig peritoneal macrophages (15). Cytophilic properties have been noted also for mouse and rat lymphocytes and polymorphonuclear leukocytes (1). Furthermore, aggregated heavy chains of H2 antigens, and to a lesser extent also monomeric chains, showed an affinity for staphylococcal protein A, competing with the immunoglobulin receptor (17).

Immunoglobulin Fc-binding properties of gram-positive cocci were first discovered in staphylococci (7) and later in streptococcus groups A, C, and G (10). Recent studies have indicated major differences in specificities of

these immunoglobulin-binding bacteria (14). Three major types of Fc-reactive structures were defined: type I, represented by staphylococcal protein A; type II, represented by streptococcus group A; and type III, represented by both group C and group G streptococci (14). In view of the similarity between the CH2 domain, carrying structures with affinity for gram-positive cocci, and β_2 -microglobulin, this latter component of the histocompatibility antigen might also show an affinity for Fc-binding gram-positive cocci. The present experiments, designed to investigate this possibility, revealed a marked reactivity between aggregated β_2 -microglobulin and structures on group A, C, and G streptococci different from immunoglobulin-binding components.

MATERIALS AND METHODS

Bacterial strains. A total of 166 strains of human pathogenic bacteria was included in the study. All strains were obtained consecutively from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospitals, Lund, Sweden, for bacteriological examination. The bacterial species and numbers of strains were as follows: *Staphylococcus aureus*, 16; *Staphylococcus epidermidis*, 11; *Staphylococcus saprophyticus*, 10; group A streptococci, 10; group B streptococci, 11; group C streptococci, 27; group G streptococci, 28; *Streptococcus faecalis*, 10; *Streptococcus*

pneumoniae, 12; *Escherichia coli*, 10; *Klebsiella pneumoniae*, 10; and *Pseudomonas aeruginosa*, 11. The strains were subcultured on blood agar plates and then grown in tryptone broth or Todd-Hewitt broth for studies of β_2 -microglobulin binding.

β_2 -Microglobulin. Human and rabbit β_2 -microglobulins were purified from urine specimens as described previously (3, 4). The proteins were radiolabeled with ^{125}I by using the chloramine-T method (8).

Aggregation of β_2 -microglobulin. Preparations of aggregated human β_2 -microglobulin were obtained by using glutaric dialdehyde as a coupling reagent, by the method of Avrameas (2). Equal volumes of β_2 -microglobulin (2 mg/ml) and 1.6% glutaric dialdehyde in 0.1 M phosphate buffer, pH 7.5, were mixed and incubated at room temperature for 1 h. One volume of 1% sodium metabisulfate was then added, and the reaction mixture was subjected to dialysis or gel filtration. No precipitate was formed during the aggregation.

Gel filtration. Aggregated β_2 -microglobulin was chromatographed on a Sephadex G-200 column (diameter, 25 mm; height, 900 mm) equilibrated with phosphate-buffered saline (0.12 NaCl-0.03 M phosphate, pH 7.3-0.02% sodium azide) containing 0.1% Tween 20. Fractions containing 3.75 ml were eluted at a speed of 12 ml/h.

Radiolabeled human IgG. Human polyclonal immunoglobulin G (IgG) (lot 62842; A. B. Kabi, Stockholm, Sweden) was radiolabeled with the chloramine-T method (8, 13) and used for measuring the F-binding capacity of strains as described previously (14).

β_2 -Microglobulin-binding assay. Overnight cultures of bacterial strains in tryptone or Todd-Hewitt broth were washed twice in phosphate-buffered saline and suspended to 10^9 bacteria per ml, as calculated from optical density at 620 nm values on diluted samples in a Beckman CP-1 calorimeter. The final suspension was made in phosphate-buffered saline with 0.1% human serum albumin (A. B. Kabi) and 0.05% Tween 20 added.

To 0.2- μg portions of β_2 -microglobulin preparations 200 μl of bacterial suspension was added and left at room temperature for 1 h. A 2-ml quantity of phosphate-buffered saline containing albumin and Tween 20 was then added, the bacteria were centrifuged, and the supernatant was discarded. Radioactivity was measured in an LKB-Wallac 1270 Rackgamma gamma radiation counter (A. B. Biotec, Stockholm, Sweden). The uptake of radiolabeled β_2 -microglobulin or human immunoglobulin was expressed as percent radioactivity remaining in the pellet.

Trypsin digestion. Increasing amounts of trypsin (catalog no. T-8253; Sigma Chemical Co., St. Louis, Mo.) were added to bacterial suspensions (10^9 bacteria per ml in 0.25 M phosphate buffer, pH 7.5), followed by incubation at 37°C for 20 min. Digestion of bacterial proteins was stopped by the addition of Trasylol (Bayer) and subsequent washings. Resuspended bacteria were then tested for uptake of β_2 -microglobulin and human immunoglobulin. In some experiments, the amount of trypsin added was kept constant and the digestion time was varied between 5 and 120 min.

Ultrasonic treatment. Samples (1 ml) of bacterial

suspensions were treated with ultrasound by using a Branson B-12 Sonifier with microtip attachment (Branson Power Co., Danbury, Conn.).

RESULTS

Monomeric β_2 -microglobulin-binding studies. Radiolabeled human and rabbit β_2 -microglobulin preparations were added to *S. aureus* strains and to group A, C, and G streptococci in binding assays. No uptake of these monomeric preparations was noted to any of the strains.

Binding of aggregated human β_2 -microglobulin to bacteria. Glutaraldehyde-aggregated, radiolabeled human β_2 -microglobulin with a relative molecular weight of 275,000 on gel filtration was added to suspensions of bacteria in binding assays. In all, 166 strains of 12 species were tested. Three species, group A, C, and G streptococci, showed marked binding of aggregated β_2 -microglobulin (Fig. 1). All 10 group A streptococci were reactive, with binding of 70 to 100% of added β_2 -microglobulin. The reactivity with group C and G streptococci was more heterogeneous, with an uptake ranging from 0 to 100% (Fig. 1). Eight bacterial species were completely negative, whereas 4 of 11 group B streptococcal strains showed slightly elevated levels.

Effect of aggregate size on binding. Aggregated human β_2 -microglobulin was gel filtered on a calibrated Sephadex G-200 column. The major part of the protein was eluted as 275,000-molecular-weight complexes, with decreasing amounts of smaller aggregates down to monomers. Fractions with different aggregate size were collected, and the binding to two group A streptococci was tested (Fig. 2). There was a marked increase in binding of β_2 -microglobulin

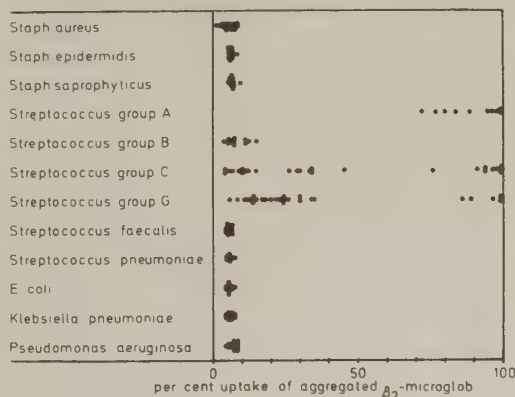


FIG. 1. Quantitative binding of aggregated β_2 -microglobulin (0.2- μg portions) to bacterial strains of 12 different species.

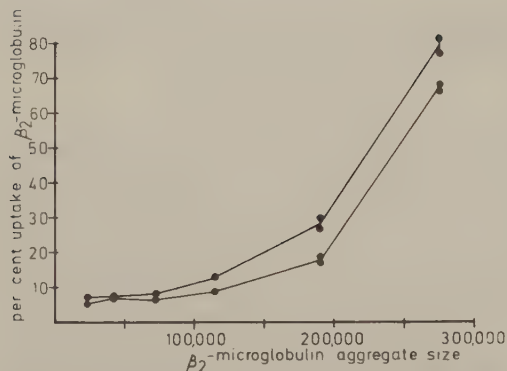


FIG. 2. Quantitative binding of β_2 -microglobulin of various aggregate sizes (0.2 μ g in each test) to two strains of group A streptococci.

with increasing molecular aggregation. Smaller aggregates below an apparent molecular weight of 100,000 showed only slight reactivity.

Effect of incubation time on binding. The influence of incubation time at room temperature on the binding of aggregated, radiolabeled β_2 -microglobulin was studied by using two group A streptococcal strains (A1 and A207) (Fig. 3). One strain (A1) showed a level of uptake characteristic of most group A strains. The other strain (A207) was included as a representative of strains with a lower level of binding. The A1 strain with a high uptake tended to reach equilibrium at a faster rate, with almost maximal binding after only 10 min of incubation. The A207 strain with a lower uptake did not reach its equilibrium even after 120 min of incubation. The results indicate that different levels of binding also seem to reflect differences in speed or strength of association rather than quantitative binding differences only.

Correlation to immunoglobulin Fc reactivity. Group A, C, and G streptococci, known to carry type II and type III Fc-binding proteins (14), were tested simultaneously for uptake of aggregated β_2 -microglobulin and normal human IgG (Fig. 4). There was a complete dissociation between the two binding properties, suggesting that different bacterial structures are involved. Three distinct groups of G streptococci were seen (Fig. 4C). One group was highly reactive with both ligands, another group was strongly IgG reactive, and a third group was IgG negative, the latter two with low binding of β_2 -microglobulin.

Inhibition experiments. Most group A, C, and G streptococci have the capacity to bind normal human immunoglobulin (10, 14) and to bind human fibrinogen as well (9, 20; G. Kronvall, C. Schönback, and E. Myhre, manuscript in preparation). Inhibition experiments were

performed to evaluate possible steric interactions between immunoglobulin and β_2 -microglobulin binding. Amounts of normal human IgG, ranging from 0.01 to 100 μ g, were mixed with aggregated, radiolabeled β_2 -microglobulin (0.2 μ g) before the addition of test bacteria in the binding assays. Two group A streptococci (A1 and A207), one group C strain (C36), and one group G strain (G22) were included, representing various types of IgG and fibrinogen reactivities. There was no influence on the uptake of β_2 -microglobulin by immunoglobulin over the entire concentration range in these inhibition experiments.

Trypsin sensitivity of β_2 -microglobulin-binding structures. Digestion experiments were performed to detect possible trypsin sensitivity of the binding structures and to compare these with the Fc-binding protein. With increasing amounts of trypsin (2.5 to 200 μ g/ml of bacterial suspension) there was a rapid fall in reactivity of group A, C, and G streptococci with aggregated β_2 -microglobulin (Fig. 5A). The loss of reactivity was almost complete when amounts as small as 20 μ g were used. On the other hand, the Fc-binding properties were completely unchanged with these amounts of trypsin over the entire range (Fig. 5B).

Effect of ultrasonic treatment on binding structures. Suspensions of two group A streptococcal strains, one group C, and one group G

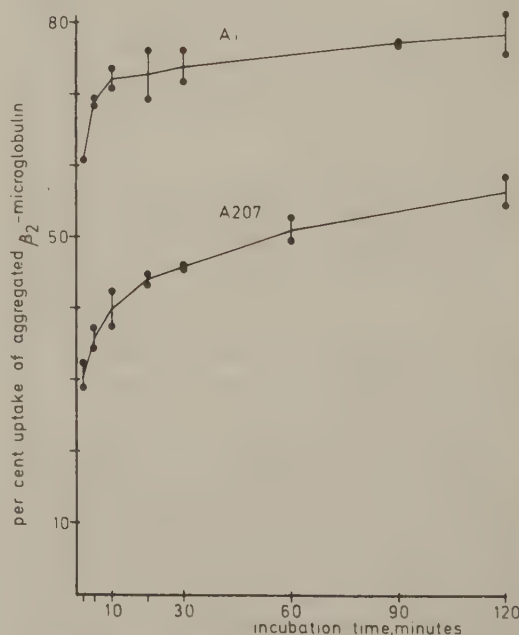


FIG. 3. Effect of incubation time on the binding of aggregated β_2 -microglobulin to group A streptococci (strains A1 and A207).

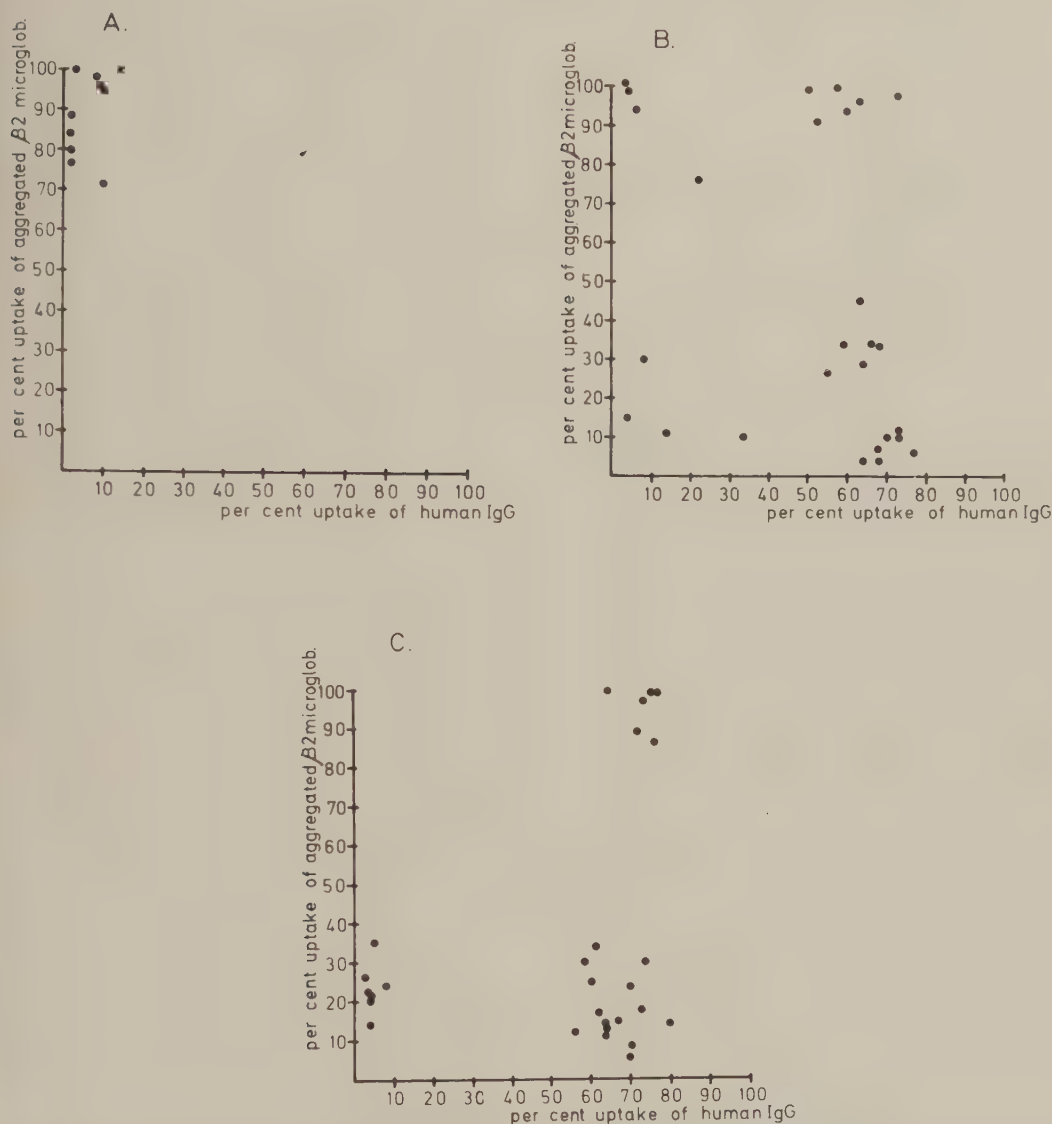


FIG. 4. Correlation between uptake of normal human IgG and aggregated β_2 -microglobulin in individual streptococcal strains of human origin. (A) Group A streptococcal strains; (B) group C streptococcal strains; (C) group G streptococcal strains.

strain were treated with ultrasound for 5, 15, and 60 s. The suspensions were then washed, and the capacity to bind aggregated β_2 -microglobulin was measured. The IgG-binding capacity was also determined for comparative purposes. Smears of the suspensions were stained with acridine orange (11), and the degree of aggregation was estimated microscopically. Both group A streptococci showed a loss of β_2 -microglobulin binding after ultrasonic treatment (Fig. 6A). The effect was most marked on the A207 strain, whereas the binding to the A1 strain was not so drastically reduced (Fig. 6A). There was no

measurable effect of ultrasonic treatment on the capacity of strain C36 or G22 to bind aggregated β_2 -microglobulin. In control experiments where the uptake of normal human immunoglobulin was measured, there were no effects noted under the present conditions of ultrasonic treatment (Fig. 6B).

DISCUSSION

Structural and functional similarities between β_2 -microglobulin and the constant domains of human IgG prompted the present investigation of possible interactions between β_2 -microglobu-

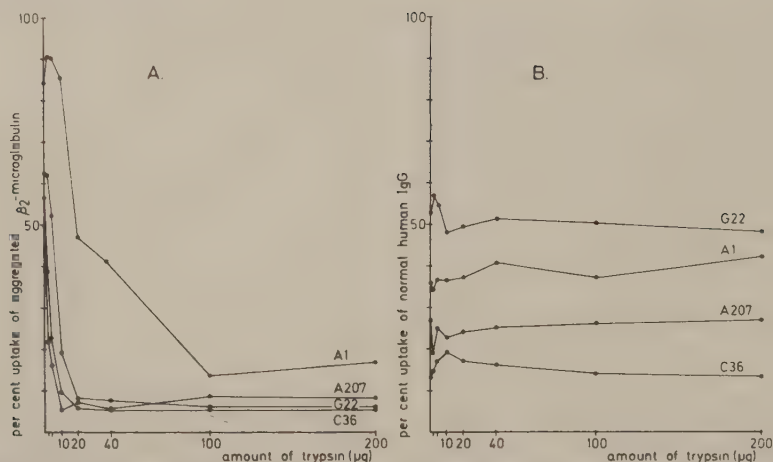


FIG. 5. Effect of trypsin digestion of streptococcal strains on the capacity to bind aggregated human β_2 -microglobulin (A) or normal human IgG (B).

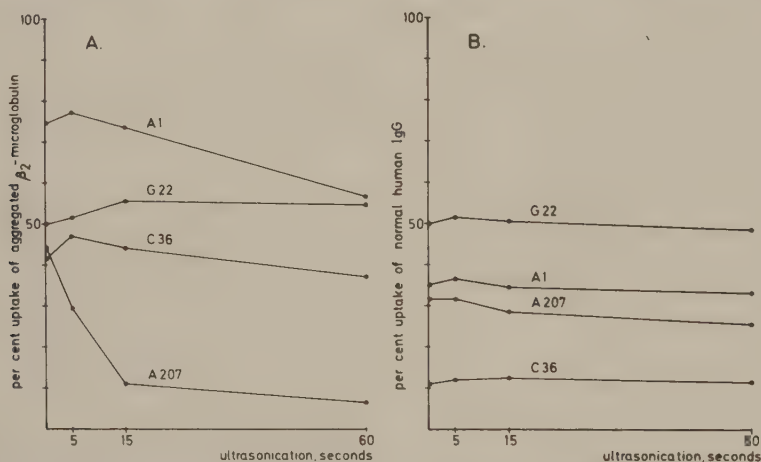


FIG. 6. Effect of ultrasonic treatment on the binding of aggregated β_2 -microglobulin (A) and normal human IgG (B).

lin and Fc-reactive, gram-positive cocci. Bacteria with defined IgG-binding properties were included, as well as other bacterial species. Binding of monomeric as well as aggregated β_2 -microglobulin was tested for 166 strains belonging to 12 different species (Fig. 1). Only group A, C, and G streptococci showed reactivity with β_2 -microglobulin, and only aggregates of β_2 -microglobulin with apparent molecular weights above 100,000 showed significant binding.

The most striking feature of the group A, C, and G streptococcal reactivity with aggregated β_2 -microglobulin was the lack of correlation with Fc-binding structures type II and type III in the same bacterial species. This was shown in direct studies of binding with a complete lack of correlation (Fig. 4), in inhibition experiments with no effect of added IgG, and in differences in

trypsin and ultrasound sensitivity of the bacterial surface proteins involved (Fig. 5 and 6). Therefore, the results of our studies do not suggest any direct similarity between β_2 -microglobulin and IgG CH2 domain structures with binding affinity for gram-positive bacteria. Such a similarity has previously been proposed to exist between H-2 heavy chains and immunoglobulin (17). We have discovered a surface marker on group A, C, and G streptococci different from immunoglobulin-binding structures.

When group A, C, and G streptococci were compared with each other concerning the characteristics of β_2 -microglobulin binding, the group A streptococci appeared different from group C and G streptococci. Group A streptococci were all highly reactive (Fig. 1 and 4A). Ultrasonic

treatment caused a significant decrease in the reactivity of two group A strains, whereas one group C strain and one group G strain were unaffected. Group C and G streptococci segregated into one highly reactive group and one low- or nonreacting group. Immunoglobulin Fc-binding characteristics show a similar correlation with a type II reactivity defined in group A streptococci and a type III reactivity among both group C and group G streptococci. The nonidentity of β_2 -microglobulin binding and IgG Fc binding has been demonstrated by other tests. The similar correlation to streptococcal groups might therefore only reflect a more recent evolutionary divergence of group C and G streptococci from the common origin of all three group A, C, and G species.

The new surface marker on group A, C, and G streptococci, detected by the binding of aggregated β_2 -microglobulin, seems to be a protein, as judged by trypsin digestion experiments (Fig. 5). The marked trypsin sensitivity is similar to that of M-proteins and differs from that of other streptococcal surface proteins, such as the T-antigens and the R-antigens. A direct association with M-protein structures is therefore possible. This might explain the reported M-protein-associated adherence of streptococci to epithelial cells (6).

Unlike immunoglobulin Fc and fibrinogen binding, there is no binding of the monomeric form of the ligand to the β_2 -microglobulin-reactive protein in the present test system. In vivo, β_2 -microglobulin is probably located on all nucleated cells. Its function might therefore be associated with a nonsoluble state. The emergence of a receptor on streptococci might be due to an evolutionary pressure on a reactivity with cell membrane-bound, nonsoluble, functionally aggregated β_2 -microglobulin. The possibility of false-negative results with monomeric β_2 -microglobulin in the assay should also be considered. If the binding site on β_2 -microglobulin is sensitive to iodination, only aggregates containing both intact and ^{125}I -labeled molecules would reveal a binding in the test system. Aspects of the reactive structure on β_2 -microglobulin are currently being explored.

In the present investigations we have defined a surface protein structure on group A, C, and G streptococci with reactivity for aggregated β_2 -microglobulin. This interaction adds a new type of mammalian-microbial short circuit to some other types of interactions known to occur with group A, C, and G streptococci. Fibrinogen will bind to group A streptococci (20), to their M-protein (9), and also to group C and G streptococci (Kronvall et al., manuscript in prepara-

tion). IgG will bind to group A streptococci (Fc-binding protein type II) and to group C and G streptococci (Fc-binding protein type III) (10, 14).

The existence of a range of possible interactions between mammalian proteins and bacterial surface structures suggests an important role of short circuits in host-parasite relations (12).

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VII

β_2 -MICROGLOBULIN IS BOUND TO STREPTOCOCCAL M PROTEIN

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Abbreviations:

β_2 m: β_2 -Microglobulin

M+ and M-: M protein-positive and M protein-negative

HLA will collectively refer to HLA-A, B and C antigens

SUMMARY

Presence of receptors for three human proteins (fibrinogen, IgG and β_2 -microglobulin) was studied in group A streptococcal strains, M type 1, 12 and 14, and in M protein-negative variants of these strains. Fibrinogen binding was detected in all six strains. IgG did not bind to the two type 12 strains and uptake was low in both type 14 strains. The type 1, M protein-positive strain, was negative in binding experiments with radiolabelled IgG, whereas the M protein-negative variant was strongly positive. Aggregated β_2 -microglobulin showed a high degree of reactivity with the M protein-positive strains, but not with the corresponding M protein-negative variants. The results indicate that the receptor for aggregated β_2 m is located on M protein in group A streptococci.

INTRODUCTION

β_2 -microglobulin (β_2m) was originally isolated from urine by Berggård and Bearn (2). This low-molecular-weight protein is also present in other biological fluids (2) and on the surface of all nucleated cells (19). At the cell surface, β_2m occurs linked to the HLA-A, B and C antigens as the invariant light chain of these molecules (10, 18, 21). Amino acid sequence analysis has revealed homology between β_2m and immunoglobulin domains (20, 23). This prompted us to investigate the possible interaction between β_2m and Fc-reactive bacteria. Many streptococcal strains were indeed found to bind aggregated β_2m (12). This binding was, however, not mediated by streptococcal Fc receptors but rather through other bacterial surface structures (12, 13).

β -hemolytic streptococci group A carry M protein antigens on their surfaces which play a central role in the pathogenicity of these microorganisms. As a matter of fact, M protein antigens seem to be the major virulence factor of group A streptococci by permitting them to colonize a susceptible host (for M protein references see review, ref. 7). The existence of M protein-positive (M+) and M protein-negative (M-) variants of group A streptococci offered an opportunity to investigate if M protein is also responsible for the binding of some human serum proteins known to interact with streptococcal surface structures.

MATERIALS AND METHODS

Streptococcal strains

Six strains of β -hemolytic streptococci group A were used in this study: Type 1; T1V without (T1-) and T1V with M protein (T1+) activity were kindly supplied by Dr. H.E. Beachey. Type 12; NY5 strain without (T12-) and with (NY5 strain after mouse passages; code E14/51RB/21/2) M protein activity (T12+) were R. Lancefields' reference strains kind-

ly supplied by Dr. Waleria Hryniewicz, National Institute of Hygiene, Warsaw. Type 14 strains (T14-: 33RS84, T14+: PHLS Lowe) were also supplied by Dr. W. Hryniewicz, Warsaw. The bacteria were grown in Todd Hewitt broth.

Proteins

β_2 -microglobulin was purified from human urine as described previously (2) and labelled with 125-iodine using the chloramine-T method (9). The protein was aggregated by the method of Avrameas (1) by adding 80 μ l of 0.25% glutaric dialdehyd in distilled water to 400 μ l of radiolabelled β_2 m (10-11 mg/ml) in 0.15 M phosphate buffer, pH 7.6. The mixture was incubated over night at room temperature and then subjected to gel-filtration on Sephadex G-200. Material corresponding to a molecular weight of about 300.000 was pooled and used in binding experiments.

Human fibrinogen and IgG (AB Kabi, Stockholm, Sweden) were radiolabelled with 125-iodine using lactoperoxidase as oxidising agent (16).

Binding assays

Overnight broth cultures of bacterial strains were washed twice in phosphate-buffered saline, pH 7.2, containing 0.02% sodium azide (PBSA). The optical density at 620 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 1×10^9 organisms per ml of PBSA containing 0.1% human serum albumin (AB Kabi) and 0.05% Tween 20 (PSAT). Bacterial suspensions (200 μ l) were incubated with aggregated radiolabelled β_2 m (0.5 μ g), fibrinogen (0.2 μ g) or IgG (0.2 μ g) at room temperature for 60 minutes. Two ml of PSAT buffer was then added and the bacteria were sedimented by centrifugation at 2000 x G for ten minutes. The radioactivity of the pellet was measured in a gamma counter and the binding expressed as percent radioactivity remaining in the pellet.

Inhibition studies

In order to obtain maximal sensitivity in inhibition experiments varying amounts of bacteria were tested for binding of aggregated β_{2m} . Depending on which bacterial strain that was investigated, bacterial dilutions containing between 2×10^8 and 5×10^8 bacteria per 200 μ l PSAT were used. Varying amounts of unlabelled fibrinogen (1-1000 μ g) was then added. Tubes were incubated at room temperature for 30 minutes followed by addition of radiolabelled aggregated β_{2m} . Tubes were subsequently incubated and processed as described for the binding assay.

RESULTS

Binding studies

The binding of radiolabelled fibrinogen, IgG and aggregated β_{2m} to three M+ group A streptococcal strains and to their M- variants was measured. The M+ strains showed a clear uptake of aggregated β_{2m} whereas this binding was almost completely lost for the M- type 1 and 14 strains. The M- type 12 strain had a low reactivity with β_{2m} (Table 1). In experiments with fibrinogen the binding pattern was much less influenced by the switch from M+ to M- strains. A slight decrease in uptake was, however, recorded for M- strains type 12 and 14 as compared to the corresponding M+ strains (Table 1). Binding experiments performed with IgG and the type 1 and 14 strains suggest that M- bacteria are in fact more IgG reactive than are M+. No difference in the binding of radiolabelled IgG was, however, demonstrated for the two type 12 streptococcal strains.

Inhibition experiments

In order to investigate a possible relationship between the binding of

aggregated β_2m and fibrinogen to the strains used in this study, M+ bacteria were incubated with unlabelled fibrinogen prior to addition of radiolabelled, aggregated β_2m . As shown in figure 1 the uptake of β_2m was inhibited. Unfortunately, scarcity of material made it impossible to test if the uptake of radiolabelled fibrinogen can be inhibited by unlabelled, aggregated β_2m .

DISCUSSION

The various connections between β_2m and the immune system, especially the structural similarities between β_2m and immunoglobulins, provided a basis for our initial interest in possible interactions between β_2m and Ig-binding bacterial species. With this background it was somewhat surprising to find, that the binding of IgG and aggregated β_2m to group A, C and G streptococcal strains, did not correlate. Furthermore, the uptake of β_2m was not inhibited by IgG (12). On the other hand, a close correlation was found between the binding of β_2m and fibrinogen to these strains (13). Fibrinogen is known to have affinity for streptococcal M proteins (11, 25). Like the structures responsible for streptococcal β_2m -binding, M proteins are sensitive to trypsin treatment but extremely heat stable (12, 14, L. Björck and G. Kronvall, unpublished). In the present investigation, M+ strains of group A streptococci show a high uptake of aggregated β_2m , whereas M- variants of these strains lack or show moderate uptake. IgG, however, exhibit a quite different binding pattern. Taken together, these data suggest that β_2m and Ig-binding is localised on different bacterial structures and that streptococcal M protein mediates the binding of aggregated β_2m . Since M proteins are believed to be important antiphagocytic surface structures, primarily responsible for the virulence of group A streptococci (15), this interaction could be of significance to the host-parasite relationship.

Compared to β_2m -binding, the uptake of fibrinogen was much less influenced by the switch from M+ to M- strains, although this protein is reported to bind to M protein (see above). This suggests that parts of the M protein remain unchanged in M- variants. This notion is supported by previous reports that various nontypable strains produce M protein-like molecules (6, 17). Even small alterations in the protein structure and subsequent loss of critical antigenic determinants could result in a transfer of an M+ strain to an M-. Thus, when the six strains used in this study were analysed according to an experimental procedure (3) including radiolabelling, solubilisation and analysis of bacterial cell wall proteins with electrophoresis in sodium dodecyl sulphate, we could find no evidence indicating that M- strains lose any major cell wall protein component, as compared to the M+ variants (L. Björck and G. Kronvall, unpublished)

The fact, that binding of β_2m to streptococci requires aggregates of the protein indicates a possible multipoint attachment. At the cell surface such a mode of attachment could be created between multiple cell-bound β_2m molecules and adhering M protein-carrying streptococci. Ellen and Gibbons (5) reported that M+ group A streptococci attached to epithelial cells, whereas M- strains did not. In our hands, F (ab')₂ fragments prepared from anti- β_2m IgG antibodies, decrease the adherence between group A streptococci and buccal epithelial cells (L. Björck, M. Söderström and G. Kronvall, unpublished). These results suggest that an interaction between cell-bound β_2m and M proteins may be of importance for the colonization of a susceptible host.

According to the domain hypothesis the immune system has evolved from a single domain gene (4, 8). The structural similarities between β_2m ,

immunoglobulins and also the heavy chain of the major histocompatibility antigens (22, 24, 26) support this theory, which also indicates that the highly specialised immune apparatus among vertebrates was preceded by more primitive cell-to-cell recognition systems. β_2m is present at the surface of most, if not all, nucleated vertebrate cells. It is also closely related to molecules of the vertebrate immune system. If the binding of human, aggregated β_2m to streptococci reflects a cell-to-cell interaction between vertebrate cells and bacteria in vivo, this interaction might also represent a reminiscence of such primitive recognition mechanisms.

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Table 1

Binding of radiolabelled, aggregated β_2m , fibrinogen and IgG to M protein-positive (+) and M protein-negative (-) strains of β -hemolytic streptococci group A.

Per cent binding of radiolabelled protein (Mean $\pm$ S.D.)			
Strain	Aggregated β_2m	Fibrinogen	IgG
T 1 (+)	60,2 $\pm$ 2,6	47,4 $\pm$ 3,8	6,7 $\pm$ 1,3
T 1 (-)	4,2 $\pm$ 1,2	46,8 $\pm$ 2,5	81,2 $\pm$ 4,9
T 12 (+)	72,8 $\pm$ 7,4	49,7 $\pm$ 4,4	6,8 $\pm$ 1,2
T 12 (-)	14,8 $\pm$ 2,6	34,3 $\pm$ 7,8	5,4 $\pm$ 0,8
T 14 (+)	51,9 $\pm$ 12,5	47,4 $\pm$ 2,5	12,6 $\pm$ 0,3
T 14 (-)	5,5 $\pm$ 0,5	41,2 $\pm$ 2,6	20,5 $\pm$ 1,9

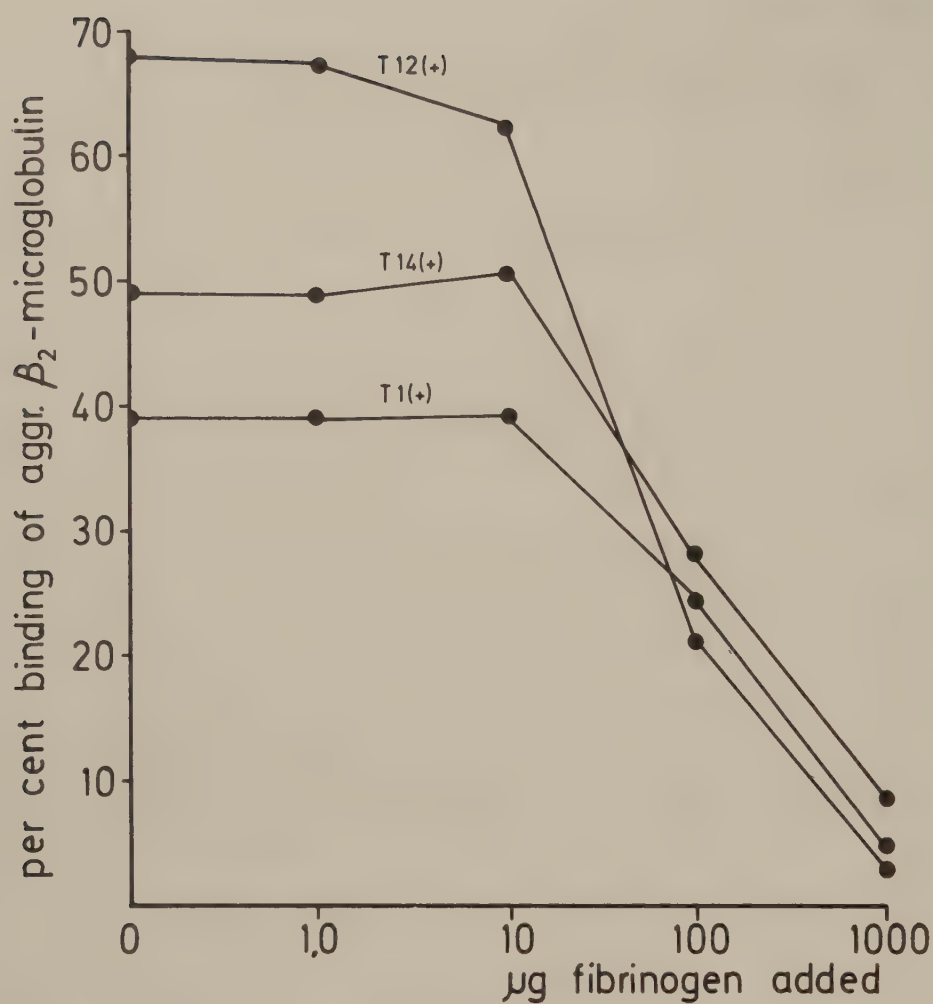


Fig. 1

Inhibition of binding of radiolabelled, aggregated β_2 m to M+ strains of β -hemolytic streptococci group A by increasing amounts of fibrinogen.

